ISBN: 978-93-88901-53-6

Research and Development in Pharmaceutical Science Volume V

Editor:
Mr. Ajit Kumar Varma
Dr. M. Shanthala
Dr. Pratibha
Dr. Pralhad Rege

Bhumi Publishing, India

First Edition: May 2023

Research and Development in Pharmaceutical Science

Volume V

(ISBN: 978-93-88901-53-6)

Editors

Mr. Ajit Kumar Varma

Faculty of Pharmaceutical Sciences,

Rama University,

Kanpur (U.P.), India

Dr. M. Shanthala

Centre for Applied Genetics,

Bangalore University,

Bengaluru (Karnataka), India

Dr. Pratibha

Faculty of Agriculture,

Madhav University, Abu Road,

(Sirohi) Rajasthan, India

Dr. Pralhad Rege

Department of Chemistry,

St. Xavier's College,

Mumbai, (M.S.), India

Bhumi Publishing

2023

First Edition: May, 2023

ISBN: 978-93-88901-53-6

Published by:

Bhumi Publishing,

Nigave Khalasa, Kolhapur 416207, Maharashtra, India

Website: www.bhumipublishing.com

E-mail: bhumipublishing@gmail.com

Book Available online at:

https://www.bhumipublishing.com/book/

PREFACE

Research and development in the field of pharmaceutical science has always been at the forefront of advancements in healthcare. It is through continuous exploration, innovation, and collaboration that we are able to discover and develop novel drugs, therapies, and treatment modalities that improve the quality of life for millions of individuals worldwide.

This book serves as a comprehensive guide to the exciting world of pharmaceutical research and development. It brings together the collective knowledge and expertise of leading scientists, researchers, and practitioners who have dedicated their careers to pushing the boundaries of pharmaceutical science. It aims to provide a comprehensive overview of the latest advancements, methodologies, and challenges in this dynamic field.

The landscape of pharmaceutical research and development is ever-evolving, driven by a growing demand for more effective, safer, and targeted therapies. From drug discovery and preclinical development to clinical trials and regulatory approval, the journey of a pharmaceutical product is complex and multifaceted. This book delves into each stage of this journey, offering valuable insights and practical guidance for researchers, professionals, and students alike.

The chapters in this book cover a wide range of topics, including drug design and synthesis, pharmacokinetics and pharmacodynamics, formulation development, drug delivery systems, biopharmaceutical analysis, and regulatory considerations. The contributors have meticulously curated their expertise to provide readers with a comprehensive understanding of the multidisciplinary nature of pharmaceutical research.

Moreover, this book recognizes the pivotal role of collaboration and knowledge sharing in driving scientific progress. It highlights the importance of interdisciplinary approaches, bridging the gap between academia, industry, and regulatory bodies to accelerate the translation of scientific discoveries into meaningful therapeutic interventions.

Editors

TABLE OF CONTENT

FORMULATION DEVELOPMENT OF HERBO-COSMETIC EMULGEL CONTAINING VIGNA MUNGO SEED EXTRACT

R. Prabhu*, B. Gowsalya, A. Iswarya, N. Kathiresan and K. Kaviya
Department of Pharmaceutics,
College of Pharmacy,
Madurai Medical College,
Madurai 625020 (TN), India.
*Corresponding author E-mail: prabhurajaram2005@gmail.com

Abstract:

Herbo-cosmetics are defined as cosmetic products containing one or more herbal ingredients. Nowadays herbo- cosmetic products are of greater interest by consumers in the market due to their least side effects. Emulgel is defined as emulsion of oil in water or water in oil type which is gelled with the help of gelling agent. Emulgel system combines the benefits of both emulsion (cream) and gel. It delivers the active ingredient to site of action by having better penetration properties. Vigna mungo seeds contain flavonoids, saponins, tannins, alkaloid, vitamin C, steroids. The seeds possess antioxidant, anti-aging and skin whitening properties. The ethanolic extract of Vigna mungo seeds are known to possess antioxidant and anti-tyrosinase activity. Antioxidants such as vitamin C, phytoextracts, reduce dopaquinone (DOPA), prevent the formation of free radicals. Antioxidant molecules employed also have skin whitening action by inhibition of tyrosinase. The products containing ethanolic extract of Vigna mungo seeds were used in the prevention and treatment of skin conditions like wrinkles, scars, photo-aging, intrinsic aging, melasma, non-melanoma skin cancer, psoriasis, and other such dermatoses. This study mainly concerns about the formulation development of herbo- cosmetic emulgel containing Vigna mungo seed extract and antioxidant potential studies of the product. By $DPPH^{\cdot}$ radical scavenging assay. The maximum $DPPH^{\cdot}$ radical scavenging activity was 77.77% at 120 µg/mL concentration of extracts, The IC_{50} was found to be 61.13µg/ml concentration and was compared with standard (Ascorbic acid, IC_{50} = 11.98 µg/ml concentration). By Superoxide radical scavenging activity. The maximum superoxide radical scavenging activity of extract was **55.75**% at 120 µg/mL concentration and the IC50 was 107.62 µg/mL concentration. By Phosphomolybdenum reduction activity studies: The maximum Phosphomolybdenum reduction was 95.32%at 120 µg/mL concentration and the RC_{50} was 1.45 µg/mL concentration. It was compared with the standard ascorbic acid (RC_{50} = 6.34 µg/mL concentration). By Ferric (Fe^{3+}) reducing power activity. The maximum Fe^{3+} reduction was 82.74% at 120 µg/mL concentrations and the RC_{50} was 1.72 µg/mL concentration. Anti-oxidant potential studies conclude that the product will exhibit sufficient antioxidant effect on skin by replenishing the natural antioxidant levels in the epidermis of the skin. The emulgel system was chosen to provide maximum beneficiary action of the seed extract to the skin. About 12 formulations were made by incorporating different oil phases and different grades of polymers in-order to prolong the contact time of the product on the skin and to achieve a better consistency. From which one with maximum anti-oxidant activity and stability is selected by optimization. The oil phase taken were

light liquid paraffin and castor oil. The gelling agents taken were Carbopol 934 and Carbopol 940.

Keywords: Emulgel, Vigna mungo, Antioxidants, Flavonoids, Herbo-Cosmetics, Aging.

Introduction:

Herbo-cosmetics are defined as the products which are prepared by using plant or herbal extract. Generally herbal sources are rich in antioxidant, hydrocolloid, vitamins, proteins, flavonoids, tannins and other bioactive components which are known for their anti-aging, antioxidant, emollient effects and are used for the preparation of cosmetics. Herbal preparations are cost effective and cause lesser side effects in long term use when compared with synthetic preparations. (1)

Various drug delivery systems are available such as oral, topical, parenteral, inhalation, transdermal etc. Topical drug delivery system is defined as the application of a drug containing formulation to the skin or mucous membrane to produce specific pharmacological effect on the surface of skin or within the layers of skin or mucous membrane (2). Skin act as a barrier to the delivery of many drug molecules in topical drug delivery system. Types of topical drug delivery system include external (that are spread or dispersed on the cutaneous surface) and internal (that are applied to the mucous membrane of eye, ear, nasal cavity, oropharyngeal cavity, vagina or anorectal region for local activity). Classification based on physical state solid (Powder, Aerosol, Plaster), liquid (Lotion, Liniment solution, Emulsion, Suspension, Aerosol), semi-solid (Ointment, Cream, Gel, Jelly, Suppository). Topical drug delivery system has certain advantages such as avoidance of first pass metabolism, avoidance of fluctuations in drug level, inter and intra-patient variations and attainment of local therapeutic effect with fewer side effects etc.(3)

Sometimes more than one formulation can be combined to enhance the delivery of drug; emulgel is such type of combination. It is the combination of emulsion and gel. **Emulgel** is prepared both in oil- in- water and water- in- oil type emulsion mixed with gel. Oil- in- water type is used for lipophilic drugs and water- in- oil type is used for hydrophobic drugs delivery. The emulgel have many advantages like shows thixotropic behaviour, non-greasy, easily spreadable and removable, emollient, bio-friendly, pleasing appearance, transparent and cosmetically acceptable, which also have good skin penetration and long shelf- life. The emulsion and gel preparations have their own properties. By the use of gelling agent classical emulsion can be converted in to emulgel. In this project we formulate the emulgel with herbal active ingredient. Emulgel is prepared by incorporating active ingredient containing oil in water emulsion into the gel preparation. Emulgel was prepared by using ethanolic extract of Vigna mungo seeds.

The **Vigna mungo seeds** contain flavonoids, saponins, tannins, alkaloid, vitamin C, steroids. The seeds possess antioxidant, anti-aging and skin whitening properties (4). The emulgel system was chosen to provide maximum beneficiary action of the seed extract to the skin. About 12 formulations were made by incorporating different oil phases and different grades of polymers. From which one with maximum antioxidant activity and stability was selected by optimization.

Anatomy of skin:

The skin is the largest organ of the body, responsible for about 15% of the total adult body weight. It involves in many vital functions, including protection against external physical, chemical, and biologic assailants, as well as prevention of excess water loss from the body and in thermoregulation. The skin is continuous, with the mucous membranes lining the body's surface the integumentary system is formed by the skin and its derivative structures.

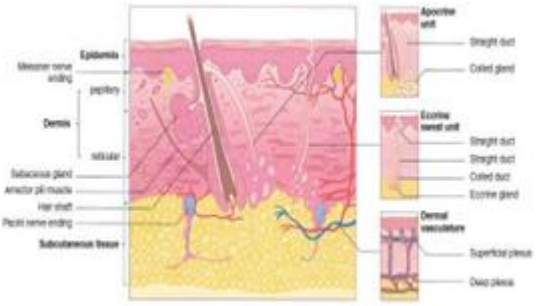


Figure 1: Structure of skin

The skin is composed of three layers:

A. **Epidermis:** The epidermis is a stratified, squamous epithelium layer that is composed of mainly two types of cells: keratinocytes and dendritic cells. The other type of cells in the epidermis includes melanocytes, Langerhans cells, and Merkel cells. The epidermis commonly is divided into four layers according to keratinocyte morphology and position as they differentiate into horny cells including
   - Basal cell layer (stratum germinativum),
   - Squamous cell layer (stratum spinosum),
   - Granular cell layer (stratum granulosum),
   - Thin, Transparent ,Glistering layer(Stratum lucidum)
   - Cornified or horny cell layer (stratum corneum) (5)

B. **Dermis:** The dermis is an integrated system of fibrous, filamentous, and amorphous connective tissue layer.(4) It provides pliability, elasticity, and tensile strength. The matrix components, including collagen and elastic connective tissue, also vary in a depth-dependent manner and undergo turnover and remodelling in normal skin, in pathologic processes, and in response to external stimuli.

C. **Subcutaneous tissue**: Subcutaneous fat is the layer of subcutaneous tissue that is most widely distributed. It is composed of adipocytes, which are grouped together in lobules separated by connective tissue. It acts as padding and as an energy reserve, as well as providing some minor thermoregulation via insulation.

Emulgel:

Emulgel are emulsions, either of the water-in-oil or oil-in-water type, which are gelled with the help of gelling agent. The emulgel system also acts as a controlled release drug delivery system in which drug particles entrapped in the system reaches the skin and slowly gets absorbed. The drug reaches the external phase of the skin in a controlled manner through the internal phases which act as a reservoir of the drug. Gel retains small drug particles and provides

its release in a controlled manner because of a cross-linked network. It prolongs the contact time of medication over the skin because of its mucoadhesive property. Since Emulgel possesses the property of both gel and emulsions it acts as a dual control release system.

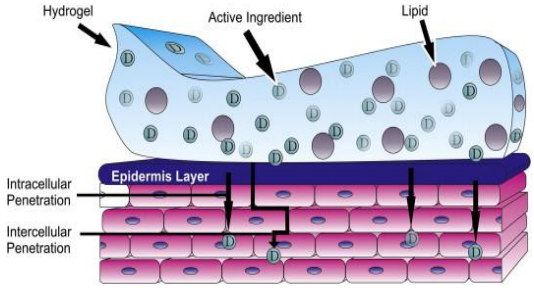


Figure 2: Emulgel formulation

Classification of emulgel:

Based on type of emulsion:

- **Water-in-oil emulsions**: They are employed for emollient actions and for the treatment of dry skin and emollient applications.
- **Oil-in-water emulsions**: They are most useful in general cosmetic as a water washable drug bases.

Ideal properties of drug candidate to formulate as emulgel:

- Drug dose should be low i.e. less than 10 mg.
- Molecular weight of drug should be 400 Dalton or less than 400 Dalton.
- Partition coefficient i.e. Log p (Octanol-water) should be between 0.4-0.8.
- Drug candidate should have a skin-permeability coefficient more than 0.5×10^{-3}cm/hr.
- Drug should be non-irritating and non-sensitizing having less polarity.(6)

Various ingredients of emulgel preparation:

1. Aqueous phase:

This forms aqueous phase of the emulsion. Generally, water is used such as demineralised or deionised water or distilled water.

2. Oils:

The oil phase significantly governs the spontaneity of the emulsification process, the droplet size of the respective emulsion and drug solubility.

Oil phases which are used in development of emulgel are balsam oil, birch oil, castor oil, isopropyl myristate, myrrh oil, rose hip oil and wheat germ oil.

3. Emulsifiers:

Emulsifiers are used to control emulsification process and stability. Stability of emulsion can be increased by emulsifiers. Surfactants having HLB values greater than 8 such as the non-ionic surfactant (ex: spans, tweens) are used in the formulation of o/w emulsions.

4. Permeation enhancer:

Penetration enhancers are the substances that assist the absorption of penetrant through the skin by temporarily thinning the impermeability of the skin. Various penetration enhancers used in the emulgel formulation are oleic acid, clove oil and menthol etc.

5. Gelling Agents:

Gelling agents are used to form gel base into which emulsion can be incorporated to form emulgel. Incorporation of gelling agent to a system makes it thixotropic. (4)

6. Preservatives:

Preservatives are agents which are used in minimal concentration in order to prolong the shelf life of the product. Ex: methyl paraben, ethyl paraben, sodium Meta bisulphite.

Advantages of emulgel:

1. Avoidance of first pass metabolism.
2. More selective to a specific site.
3. Improve patient compliance.
4. Suitability for self-medication.
5. Ability to easily terminate medication when needed.
6. Convenient and easy to apply.
7. Incorporation of hydrophobic drugs
8. Better loading capacity
9. Better stability
10. Production feasibility and low preparation cost. (4) (6).

Application of antioxidants in various disease conditions:

The following were the diseases that can be prevented by using antioxidants containing products.

Photodamage

Ultraviolet (UV) radiation, in its UVB range (290-320 nm), is responsible for the immediate damages especially on keratinocytes; UVA band (320-400 nm), which induces cellular changes, particularly by compromising melanocytes and fibroblasts. Use of antioxidants in the prevention and repair of ultraviolet Photodamage is widely studied. Association of antioxidant molecules, from vitamins to phytoextracts, in addition photoprotectors and moisturizers were used against aging. Antioxidant action of the phytoextracts occurs by the neutralizing effect of ROS, blocking lipid peroxidation, and by activating natural antioxidant systems (7).

Aging

Skin aging occurs due to decline of the endogenous antioxidant mechanisms. It is due to cutaneous atrophy, reduction of epidermis, decrease in the collagen content and other dermal elements. There will be increased ROS production, which causes cell aging. The most physiological protective effect against oxidative stress was support to the endogenous system, by using antioxidants normally present in the skin. (7)

Melasma

UV induced melanogenesis that occurs in melasma is amplified by increasing the oxidation of dopaquinone. Antioxidants such as vitamin C, phyto extracts, reduce dopaquinone (DOPA), prevent the formation of free radicals. Antioxidant molecules employed also have whitening action by inhibition of tyrosinase (7).

Non-melanoma skin cancer

Generation of free radicals induced by UV in the skin, develops oxidative stress when that exceeds the ability of natural defense: the only skin protection systems are antioxidant

enzymes and melanin, the first line of defense against DNA damage. DNA absorbs UV radiation, whose energy can break the molecular bonds; most of these breaks are repaired by enzymes present in the nucleus itself, however the remaining damages generate mutations that lead to neoplasia.

Epidermal antioxidant capacity is much higher than the dermal: catalase, glutathione peroxidase and glutathione reductase systems were higher in the epidermis than in the dermis - both the lipophilic antioxidants (tocopherol, ubiquinol 9, etc.) and the hydrophilic ones (ascorbic acid and glutathione). The stratum corneum contains both hydrophilic and lipophilic antioxidants. Vitamins C and E, as well as glutathione (GSH) and uric acid, were found. It is also confirmed that the acute exposure of human skin to UV radiation leading to oxidation can be prevented by previous treatments with antioxidants, reducing the risk of carcinogenesis.(7)

Psoriasis

The systemic signs of oxidative stress in patients with active psoriasis are plasma levels of Malonyldialdehyde (MDA) are significantly elevated, suggesting the depletion of natural enzymatic and non-enzymatic antioxidant systems and consequently the prevalence of peroxidation processes in cell membranes and plasma lipid processes of circulating cells. Similarly, SOD is reduced in erythrocytes of psoriatic patients. On the other hand, classic treatments such as phototherapy or methotrexate are also generates ROS and RNS. However, the eventual use of antioxidants should aim to recover the redox balance, leading to an anti-inflammatory effect, possibly by the activation of antiproliferative and proapoptotic pathways, both in the local and in the inflammatory cells. (7)

Other dermatoses

Many inflammation conditions cause redox imbalance and increased consumption of their antioxidant systems in local cells, such as atopic dermatitis or burned skin, and scarring process, in which the excess of ROS blocks dermal and epidermal repair. In such cases antioxidant plays a major role in maintaining the redox balance in skin.

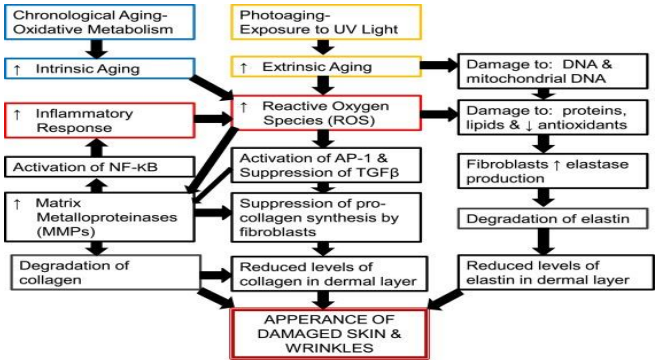


Figure 3: Mechanism behind formation of Skin damage and wrinkles

Formulation development:

The formulation of the lab made herbo cosmetic emulgel is done with the help of various emulgel related articles. Totally 12 formulations were made with certain modifications in the formula. The modifications were changes in the oil phase (liquid paraffin and castor oil) each at three levels of concentration and by using different grades of gelling agent (Carbopol 934 and

Carbopol 940) each at three levels of concentration. The best formula was selected based on better stability and physical appearance.

Table 1: Herbo-cosmetic emulgel containing Vigna mungo seed extract: (Formula)

S.NO	INGREDIENTS	USE	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
1.	DRUG EXTRACT(ml)	Active Ingredient	10	10	10	10	10	10	10	10	10	10	10	10
2.	CARBOPOL 934 (g)	Gelling Agent	0.5	1	0.75	--	--	--	0.5g	1	0.75	--	--	--
3.	CARBOPOL 940 (g)	Gelling agent	--	--	--	0.5	1	0.75	--	--	--	0.5	1	0.75
4.	LIQUID PARAFFIN(ml)	Emulsion-Oil phase	12.5	12.5	12.5	12.5	12.5	12.5	--	--	--	--	--	--
5.	CASTOR OIL(ml)	Emulsion-Oil phase	--	--	--	--	--	--	12.5	12.5	12.5	12.5	12.5	12.5
6.	ACACIA (g)	Emulsifying Agent	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125
7.	SODIUM META BISULPHITE (g)	Preservative	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
8.	TRIETHANOLAMINE(ml)	pH Adjuster	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9.	SANDALWOOD OIL (ml)	Perfume	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
10.	WATER (ml)	Solvent-Aqueous phase	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

The general procedure involved in the formulation of Herbo-cosmetic emulgel is as follows:

Procedure:

Preparation of emulsion:

- The emulsion was prepared by using wet gum method.
- The primary emulsion is to be prepared in the ratio of Oil: Water: Gum – 4:2:1.

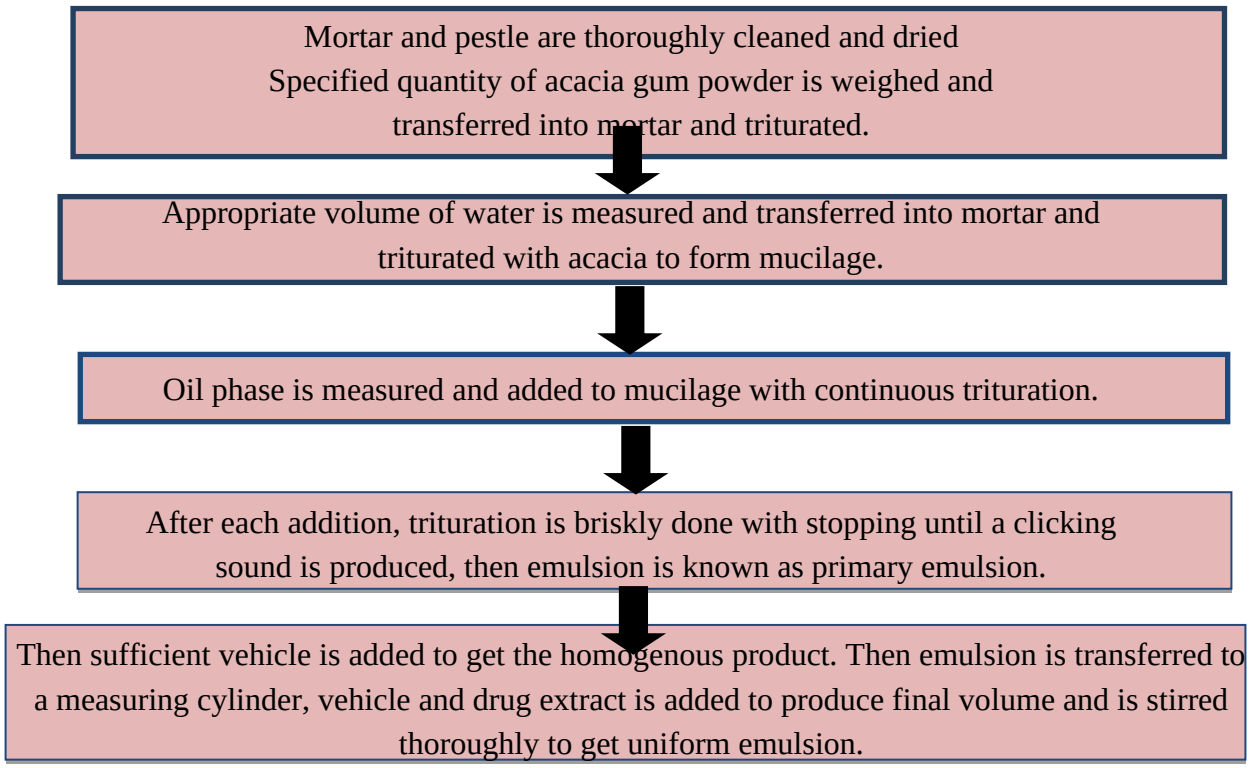


Figure 4 a: Preparation of Emulsion

Preparation of gel:

Weigh accurately required amount of gelling agent and transfer it to a beaker.

To this, add specified quantity of distilled water and stir well, until gel is formed. The pH of the gel was adjusted using Triethanolamine.

Figure 4 b: Preparation of gel

Preparation of emulgel:

To the prepared gel, add emulsion slowly and mix them well by continuous stirring to form a homogenous product. The ratio of emulsion: gel is 1:1.Then preservative and perfuming agent was added

Figure 4 c: Herbo-Cosmetic emulgel containing Vigna mungo seed extract (Our product)

Phytochemical characterisation of vigna mungo extract:

The seeds of Vigna mungo were known to contain numerous phytochemicals. In order to get necessary antioxidant and skin whitening effects, the following phytoconstituents are to be evaluated

- Tannins
- Flavonoids
- Glutathione

Chemical tests

The chemical tests were done in order to confirm the presence of tannins and flavonoids.

Detection of tannins and phenolic compounds:

- **Ferric chloride test**: 2 mL of 5% neutral ferric chloride solution were added to 1 mL of extract, the dark blue colouring indicates the presence of phenolic compounds and tannins.(8)

Detection of flavonoids:

- **Alkaline reagent test**. Two to three drops of sodium hydroxide were added to 2 mL of extract. Initially, a deep yellow colour appeared but it gradually became colourless by adding few drops of dilute HCL, indicating that flavonoids were present.(8)

Detection of glutathione:

The detection of Glutathione was done by using FT-IR spectroscopy.

Extractive value:

Weigh accurately about 5 g of coarsely powdered drug into a 250 ml conical flask with stopper. Add 100 ml of ethanol. Shake the flask frequently during first 6 hr. Keep it aside without disturbing for 18 hr. and then filter. Pipette out 25 ml of the filtrate and evaporate to dryness in a weighed shallow flat-bottomed dish on a water bath. Then dry the residue at 105^0 C to a constant weight. Calculate the percentage of ethanol-soluble extractive.

% of ethanol soluble extractive = weight of residue/weight of the drug x 100

It is expressed as per cent w/w of the air-dried drug. (9) (10)

Physical characterisation of herbo-cosmetic emulgel containing vigna mungo seed extract

1. Physical Examinations:

   The prepared emulgel formulations were inspected visually for their colour, odour, homogeneity, consistency and washability. (10) (11)

2. pH:

   1% solution in water of emulgel subjected to measure pH by the digital pH meter. (4)

3. Spreadability measurement :

   - Small amount of sample is taken and placed on the dorsum of the hand. Then it was spread evenly over the surface of the skin. The degree of ease with which the sample was spread on the skin was measured.
   - 0.5 gm of emulgel is placed on a glass slide and a circle made around it. Then a second slide is placed over it and a predetermined weight is put on it for specific time period. The increase in diameter is noted as g cm/sec.

     Spreadability = m *l/ v

   Where, S= Spreadability (g cm/sec)

m= weight placed on upper slide
l= length of the glass slide
t = time taken in sec.

4. Washability:
   Prepared formulation was added to the skin and then manually tested for ease and degree of washing with water.(10)
5. Skin Irritation Study:
   1 g of the sample was applied to an area of approximately 6cm^2 of the skin and covered with a gauze patch.(2)
6. Optimization and development of emulgel:
   Optimization was done by statistical design of the experiment.
7. Stability study of optimized batch of emulgel:
   Sample emulgel is packed in final packaging container and then kept at room temperature and relative humidity for 3 months. The physical stability could be evaluated by visual inspection for physical changes.(2)
8. FT-IR Spectroscopy:
   IR spectroscopy is an advanced analytical tool that investigates the structural chemistry of the sample by irradiating with IR radiations. Fourier transform infrared spectroscopy (FTIR) is a technique used to identify the functional groups in the materials (gas, liquid, and solid) with the help of beam of infrared radiations. The molecules or sample absorbed the IR radiations and displayed an absorption spectrum. IR measures the amount of radiations absorbed by the molecule and their intensity. The absorption of the IR radiations causes various molecular motions in the molecule, which create a net dipole moment. Therefore, a molecule is said to be IR active if the molecule has a net dipole moment (e.g., CH_4, C_2H_6, NO_2, TiO_2), otherwise it will be IR inactive (e.g., H_2,O_2, etc.). One of main advantages of FTIR spectroscopy is its capability to identify functional groups such as C=O, C-H, or N-H. FTIR spectroscopy enables by measuring all types of samples: solids, liquids, and gases.(12).

Antioxidant potential studies

Antioxidants:

Antioxidants are compound that having the ability to either delay or inhibit the oxidation processes. This reaction takes place under the presence of atmospheric oxygen or reactive oxygen species (ROS). Antioxidant involved as a defensive mechanism of the organism from an attack of free radicals.

Determination of total phenolic content:

- The total phenolic content was determined by using Folin-Ciocalteu method. Gallic acid was used as standard calibration and total phenolic content was expressed in mg Gallic Acid Equivalence (mg GAE/g).
- The total phenolic content was determined using spectroscopic method.
- The reaction mixture was prepared by mixing 1 mL plant extracts (1mg/mL), 1 mL of 10% Folin-Ciocalteu's reagent dissolved in 13 mL of deionized water followed by the addition of 5 mL of 7% Na_2CO_3 solution.

- The mixture was mixed thoroughly and kept in the dark at room temperature for 2 h. The blank solution was also prepared.
- The absorbance was recorded using spectrometer at 760 nm. All the analysis was repeated three times and the mean value of absorbance was obtained.
- Total phenolic content was determined by extrapolating calibration line which was construed by Gallic acid solution.
- The TPC was expressed as Gallic Acid Equivalent (mg GAE) per gram of the dried sample. (12)

Determination of total flavonoid content:

- The total flavonoids content of Vigna mungo was determined by using aluminium chloride calorimetric method.
- 0.5 mL of sample (1mg/mL) was mixed with 1mL of 10% aluminium chloride, 1mL of potassium acetate (1M) and 2.5 mL of distilled water.
- Quercetin was used to make the calibration curve.
- The absorbance of the mixtures was measured at 415 nm by using UV-spectrophotometer. The total flavonoid content was expressed in terms of quercetin equivalent (mg QE/g of sample). All the analyses were repeated three times and the mean value of absorbance was obtained.
- To determine the level of total flavonoid compounds in the sample, quercetin (QE) is used as a standard solution. The absorbance value of the quercetin standard solution was obtained at each concentration. A graph of the concentration curve versus the absorbance of the linear line equation was obtained.

Antioxidant assays:

1. DPPH˙ radical scavenging activity:

The antioxidant activity of the extract was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 ml of various concentrations (50-300 μg/mL) of the extract. The mixture was then allowed to stand for 30 min incubation in dark. Ascorbic Acid was used as the reference standard. One mL methanol and 1 mL DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis Spectrophotometer at 517 nm. The percentage of inhibition was calculated using the following formula:

% of DPPH˙ radical inhibition= Control – Sample/Control ×100

2. Superoxide radical scavenging activity:

The reaction mixture contains different concentrations of the extract,50 mM of phosphate buffer (pH 7.8), 1.5 mM of riboflavin, 12 mM of EDTA and 50 mM of NBT, added in that sequence. The reaction was started by illuminating the reaction mixture for 90sec. Immediately after illumination, the absorbance was measured at 590 nm and the IC_{50} was calculated. Ascorbic acid was used as positive control.

% of Superoxide radical inhibition=Control-Sample/Control×100

3. Phosphomolybdenum reduction assay:

The plant extract with concentrations ranging from 20 to 120 µg/mL was combined with reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in water bath at 90ºC for 30 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic Acid was used as standard reference. The percentage of inhibition was calculated using the following formula:

% of Phosphomolybdenum reduction = (Sample – Control / Sample)×100

4. Ferric (Fe^{3+}) reducing power assay:

The reducing power of the extract was determined by slightly modified method of Yen and Chen(2018) . One ml of plant extract of different concentrations (20 - 120 µg/mL) was mixed with 1 ml phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1 % (w/v) potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were then incubated at 50°C for 30 min. One mL of 10%(w/v) trichloroacetic acid was added to each mixture. Then to the 1 ml mixture of 0.1%(w/v) $FeCl_3$ was added and the absorbance was measured at 700 nm using Spectrophotometer. Ascorbic Acid was used as the standard reference. The percentage of inhibition was calculated using the following formula:

% of Fe^{3+} reduction = (Sample – Control / Sample) x 100

Results and discussions:

Phytochemical characterisation of vigna mungo seed extract:

1. Detection of Tannins and Flavonoids:

Table 2: Chemical tests

S.No	Test	Observation	Inference
1.	Ferric chloride test	Appearance of dark blue colour.	**Presence of phenolic compounds and tannins**.
2.	Alkaline reagent test	Initially, a deep yellow colour appeared but it gradually became colourless by adding few drops of dilute HCL.	**Presence of Flavonoids.**

2. Detection of Glutathione: FT-IR spectroscopy

The FT-IR spectra of extract are shown in Figure 13. The absorption band at 3615 and 3336 cm–1 represent the stretching frequency of N-H and –OH functional group present in the glutathione extract. The band at 2972, 2926 and 2883 cm–1 represent the glutathione Cα-H symmetric and asymmetric stretching vibration are present in the drug molecule respectively. The absorption band position at 1656 cm–1 C=O vibration of –COOH functional group and 1417 cm–1 represent the C-O-H…O (amide) vibration are present in the glutathione molecule. The band position at 1273 cm–1 suggested that C-N-H vibrations are present in the glutathione molecule [41].

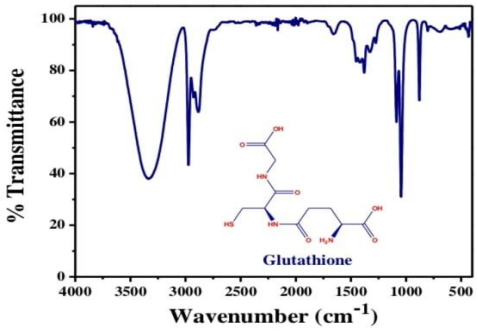


Figure 5: FT-IR Spectra of ethanolic extract of Vigna mungo seeds

3. Extractive value:

The extractive value of ethanolic extract of Vigna mungo seeds was found to be **3.3% w/w of air dried drug**.

Physical characerisation of herbo-cosmetic emulgel containing vigna mungo seed extract:

1. Physical evaluation:

Table 3: Physical evaluation of the product

S.No	Characteristics	Observation
1.	Colour	Beige or light brown
2.	Odour	Pleasant odour
3.	Consistency	Creamy, non-greasy
4.	Homogeneity	Good
5.	Spreadability	Good
6.	Washability	Good

2. pH:

The pH of the formulation was found to be **5**

3. Spreadability:

The spreadability of the formulation was found to be **13.3 g.cm/sec.**

4. Skin Irritation Study:

No allergic symptoms like inflammation, redness, irritation was observed on the area of sample application.

5. Optimization of the product:

Totally there were 12 formulations which were made by using different grades of gelling agent and different oil phases.

Optimization by factorial design:

- Factor:

Gelling agent: Carbopol 934 and Carbopol 940

Oil phase: Liquid paraffin and Castor oil.

- Level: 3 levels- High, Intermediate and low concentration.

Among 12 formulations, **Formulation 12 (F12)** is selected as a best formulation based on better physical characteristics.

6. Stability studies:

After 3 months of storage, the product was evaluated. It was observed that there was no change in the physical appearance of the product.

7. FT-IR spectroscopy of formulation:

Figure 6 reveals that FT-IR spectra of Carbopol 940 drug molecule. The O-H stretching frequency present in the absorption bands at 3450 cm–1 which confirmed the Carbopol 940 molecule. The C-H stretching, C=O stretching, C-O stretching and C-C stretching are observed position at 2930, 1694, 1412 and 1450 cm–1 which functional group are present in the Carbopol 940 [13].

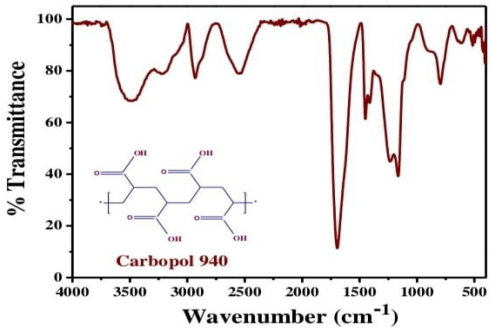


Figure 6: FT-IR Spectra of Carbopol 940

Figure 7 represent the formation of glutathione extract and carbopol 940 drug molecule in this figure, it represents that no absorption peaks or interaction observed between each other which clearly says that no interaction are obtain in the drug molecules, further evidence are shown in Figure 8.

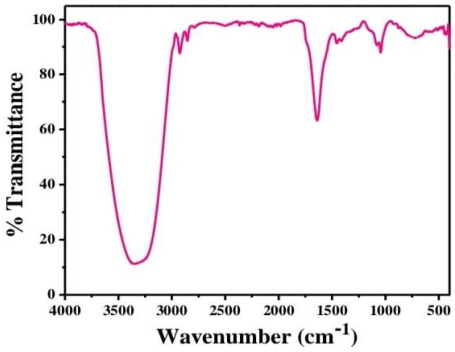


Figure 7: FT-IR Spectrum of Formulation of extract

The major functional group absorption bands are position at 3345, 2925, 2854, 1639 and 1412 cm–1 which is clearly mention that no interaction happened while doing formulation. The Figure 8 represents the overall spectral view of the drug molecule.(13) (14) (15) (16)

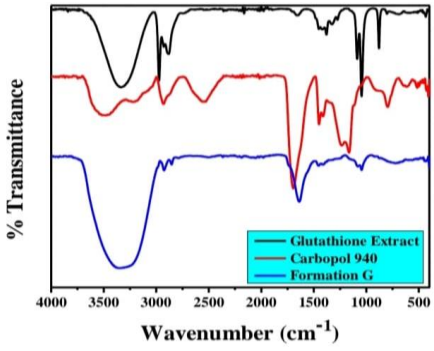


Figure 8: FT-IR Spectra of overall formulation view

Antioxidant potential studies:

1. Total Phenolic Content of seed extract and formulation:

Working standard: 100mg of Gallic Acid is dissolved in 100ml of DMSO in a standard flask.

Concentration =1mg/ml

Table 4: Total Phenolic Content

S. NO	REAGENTS	B	S1	S2	S3	S4	S5	TA 1	TA 2	TA 3	TB 1	TB 2	TB 3
1	Volume of standard	-	1.0	2.0	3.0	4.0	5.0	-	-	-	-	-	-
2	Concentration of standard	-	1.0	2.0	3.0	4.0	5.0	-	-	-	-	-	-
3	Volume of Sample	-	-	-	-	-	-	1.0	1.0	1.0	1.0	1.0	1.0
4	Volume of Distilled water	14.0	13.0	12.0	11.0	10.0	9.0	13.0	13.0	13.0	13	13	13
5	Volume of Folin's Reagent	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
6	Volume of Na2CO3	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
	The mixture kept in Dark for 2 Hours												
8	Absorbance at 760nm	0.00	3.5	7.0	10.6	14.0	17.7	3.60	3.60	3.59	2.80	2.80	2.80

Where

B – Control

S1, S2, S3, S4, S5- Standard

TA1, TA2, TA3- Sample extract (Test)

TB1, TB2, TB3- Sample formulation (Test)

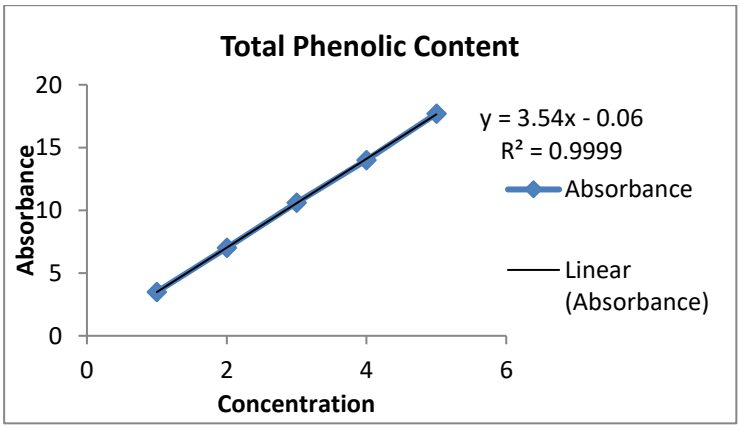


Figure 9: Calibration curve of Gallic Acid

From the graph, the linear regression equation was found to be y= 3.54x – 0.06 while the correlation was found to be 0.9999. The amount of phenol content present was calculated using the regression equation and the amount is expressed in terms mg GAE/g of extract. It was found to be 0.1144 for ethanolic extract of Vigna mungo seeds and 0.0896 for herbo cosmetic emulgel containing Vigna mungo seed extract (product) by using the above linear regression equation.

Result:

- The total phenolic content of Vigna mungo seed extract was found to be **0.1144 mg GAE /g of the sample extract.**
- The total phenolic content of the emulgel formulation was found to be **0.0896 mg GAE /g of the emulgel formulation.**

2. Total Flavonoid Content of Seed extract and Formulation:

Working standard: 100mg of Quercetin is dissolved in 100ml of DMSO in a standard flask.

Concentration =1mg/ml

Table 5: Total Flavonoid Content

S. NO	REAGENTS	B	S1	S2	S3	S4	S5	TA 1	TA 2	TA 3	TB 1	TB 2	TB 3
1	Volume of standard	-	0.1	0.2	0.3	0.4	0.5	-	-	-	-	-	-
2	Concentration of standard	-	100	200	300	400	500	-	-	-	-	-	-
3	Volume of Sample	-	-	-	-	-	-	0.5	0.5	0.5	0.5	0.5	0.5
4	Volume of Distilled water	3.0	2.8	2.0	2.5	1.0	2.0	2.5	2.5	2.5	2.5	2.5	2.5
5	Volume of AlCl3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
6	Volume of Potassium Acetate	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
7	Absorbance at 415 nm	0.00	1.29	1.58	3.87	5.13	6.43	1.67	1.67	1.70	1.19	1.19	1.19

Where

B – Control

S1, S2, S3, S4, S5- Standard

TA1, TA2, TA3- Sample extract (Test)

TB1, TB2, TB3- Sample formulation (Test)

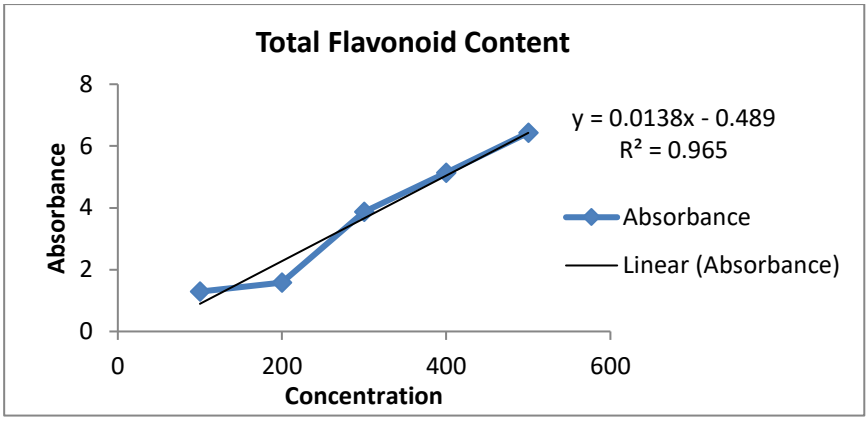


Figure 10: Calibration curve of Quercetin

From the graph, the linear regression equation was found to be y= 0.0138 x - 0.489 while the correlation was found to be 0.965. The amount of flavonoid content present was calculated using the regression equation and the amount is expressed in terms mg QE/g of extract. It was found to be 17.45 for ethanolic extract of Vigna mungo seeds and 13.44 for herbo cosmetic emulgel containing Vigna mungo seed extract (product) by using the above linear regression equation.

Result:

- The total flavonoid content of Vigna mungo seed extract was found to be **17.45 mg QE /g of the sample extract.**
- The total flavonoid content of the emulgel formulation was found to be **13.44 mg QE /g of the emulgel formulation.**

3. Antioxidant Assays:

I. **DPPH˙ radical scavenging assay :**

The maximum DPPH˙ radical scavenging activity was **77.77%**at120 µg/mL concentration of *extracts*,The IC_{50} was found to be **61.13µg/ml** concentration and was compared with standard (Ascorbic acid, IC_{50} = **11.98 µg/ml** concentration).

Table 6: DPPH˙ radical scavenging activity of Extract

S.no	Concentration (µg/mL)	% of Inhibition
1.	20	0.216
2.	40	8.83
3.	60	30.09
4.	80	49.07
5.	100	54.629
6.	120	**77.77**

II. **Superoxide radical scavenging activity**

The maximum superoxide radical scavenging activity of extract was **55.75**% at 120 µg/mL concentration and the IC50 was **107.62 µg/mL** concentration.

Table 7: Superoxide radical scavenging assay of extract.

S.no	Concentration (µg/mL)	% of Reduction
1.	20	26.54
2.	40	31.85
3.	60	36.28
4.	80	38.93
5.	100	44.24
6.	120	**55.75**

III. **Phosphomolybdenum reduction activity:**

The total antioxidant activity of the extracts was measured by Phosphomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm.The maximum Phosphomolybdenum reduction was **95.32%**at 120 µg/mL concentration and the RC_{50} was **1.45 µg/mL** concentration. It was compared with the standard ascorbic acid (RC_{50} = **6.34 µg/mL** concentration).

PM assay is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand. It involves in thermally generating auto-oxidation during prolonged incubation period at higher temperature.

Table 8: Phosphomolybdenum reduction activity of extract

S.no	Concentration (µg/mL)	% of Reduction
1.	20	68.35
2.	40	82.33
3.	60	92.37
4.	80	92.75
5.	100	93.16
6.	120	95.32

IV. **Ferric (Fe^{3+}) reducing power activity:**

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the filtered and unfiltered extracts subsequent formation of ferro-ferric complex. The reduction ability increases with increase in concentration of the extract.

The maximum Fe^{3+} reduction was **82.74%** at 120 µg/mL concentration and the RC_{50} was **1.72 µg/mL** concentration. It was compared with the standard ascorbic acid (RC_{50} = **7.72µg/mL** concentration).

Also in this assay, higher absorbance of the reaction mixture indicates higher reduction potential. The reducing capacity of extracts poses as a significant indicator of its potential antioxidant activity. The reducing capacity of the extract was performed using Fe^{3+} to Fe^{2+} reduction assay as the yellow colour changes to green or blue colour depending on the concentration of antioxidants.

Table 9: Ferric (Fe^{3+}) reducing power activity of Extract

S.no	Concentration (µg/mL)	% of Reduction
1.	20	58.31
2.	40	70.80
3.	60	70.86
4.	80	77.40
5.	100	80.28
6.	120	82.74

Conclusion:

The skin is the site of multiple oxidative reactions, neutralized by exogenous or endogenous enzymatic and non-enzymatic systems present in the skin. Epidermal cells interact with environment and are largely responsible for this generation of reactive species. Any change in this balance may induce dermatoses and the use of antioxidants may be of great value, if administered topically. Each molecule with antioxidant action has actions in certain sites and, in smaller doses, known to be more efficient. In addition, these allow administering concentrations closer to the physiological ones, which reduces toxicological risks. Herbo-Cosmetic emulgel is prepared using herbal ingredients thereby reducing the toxic side effects. As the antioxidant potential studies concluded that the product was known to have a good antioxidant action on skin, which on continuous usage reduces the chances of skin aging, wrinkles and other dermatoses. Comparing with the marketed products of Vigna mungo, the formulation was known to have better physical properties such as less greasiness, gel like consistency etc, which makes the product user-friendly. It can be concluded that herbo-cosmetic emulgel containing Vigna mungo seed extract has significant antioxidant effects and skin whitening effect on human skin.

References:

1. Yapar EA. Herbal cosmetics and novel drug delivery systems. Indian Journal of Pharmaceutical Education and Research. 2017; 51(3):152-8.
2. Patel BM, Kuchekar AB, Pawar SR. Emulgel Approach to Formulation Development: A Review. Biosciences Biotechnology Research Asia. 2021 Oct 27; 18(3):459-65.
3. Panwar A, Upadhyay N, Bairagi M, Gujar S, Darwhekar G, Jain D. Emulgel: A review. Asian J Pharm Life Sci. 2011; 2231:4423.
4. Sreevidya VS. An overview on emulgel. International Journal of Pharmaceutical and Phytopharmacological Research. 2019; 9(1):92-7.
5. Goodwin C. Anatomy and Physiology of the Skin. Journal of the Dermatology Nurses' Association. 2011; 3(4):203-13.
6. Ashara K, Soniwala M, Shah K. Emulgel: A novel drug delivery system. Journal of Pakistan association of dermatologists. 2016; 26(3):244-9.
7. Addor FA. Antioxidants in dermatology. Anaisbrasileiros de dermatologia. 2017 May; 92:356-62.
8. Kancherla N, Dhakshinamoorthi A, Chitra K, Komaram RB. Preliminary Analysis of Phytoconstituents and Evaluation of Anthelminthic property of Cayratia auriculata (In vitro). Maedica. 2019 Dec; 14(4):350.

9. https://labmonk.com/determination-of-water-soluble-extractive-value-of-ginger
10. Ms.Varsha Suryavanshi, Dr.Neelesh Chaubey Dr.C.K.Tyagy, Dr.Anurag Jain, 5Mr. FirozKhan: Formulation and evaluation of wound healing herbal gel of Bellis perennis flower extract: International Journal of Creative Research Thoughts (IJCRT) | Volume 10, Issue 9 2022 704-716.
11. Khullar R, Saini S, Seth N, Rana AC. Emulgels: a surrogate approach for topically used hydrophobic drugs. Int J Pharm Bio Sci. 2011 Jul; 1(3):117-28.
12. Khan SA, Khan SB, Khan LU, Farooq A, Akhtar K, AsiriAM. Fourier transform infrared spectroscopy: fundamentals and application in functional groups and nanomaterials characterization. Handbook of materials characterization. 2018:317-44.
13. Tamasi G, Bonechi C, Donati A, Leone G, Rossi C, Cini R, Magnani A. Analytical and structural investigation via infrared spectroscopy and density functional methods of Cuprous complexes of the antioxidant tripeptide glutathione (GSH). Synthesis and characterization of a novel CuI-GSH compound. Inorganica Chimica Acta. 2018 Jan 30; 470:158-71.
14. Abraham SA, Yashavanth G, Deveswaran R, Bharath S, Azamathulla M, Shanmuganathan S. Honey based hydrogel as delivery system for wound healing Materials Today: Proceedings. 2022 Jan 1; 49:1709-18.
15. Khan MS, Yusufzai SK, Rafatullah M, Sarjadi MS, Razlan M. Determination of total phenolic content, total flavonoid content and antioxidant activity of various organic crude extracts of Licuala Spinosa leaves from Sabah, Malaysia. ASM Sci J. 2018; 11:53-8.
16. Hilma R, Herliani H, Almurdani M. Determination of Total Phenolic, Flavonoid Content And free Radical Scavenging Activity of Ethanol Extract Sawo Stem Bark (ManilkaraZapota (L.)). Prosiding CELSci Tech. 2018 Oct 19; 3:62-8.

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) METHOD FOR ESTIMATION OF CANAGLIFLOZIN IN ITS PHARMACEUTICAL DOSAGE FORM

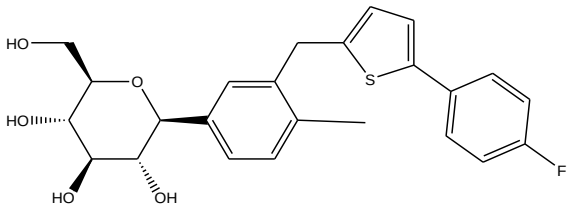
Nirav Panchal *, Pratik Tailor and Ashish Mishra
Maliba Pharmacy College,
Uka Tarsadia University, Gopal-Vidyanagar, Maliba Campus, Surat (Gujarat) - 394350, India
*Corresponding author E-mail: panchalnirav93@gmail.com

A specific, accurate, precise, and robust stability indicating HPTLC method for Canagliflozin(CNG) has been developed and validated. The separation was performed on an aluminium plate pre-coated with silica gel 60F254 using ethyl acetate: toluene: methanol (2:6:2 v/v/v) as mobile phase. After the development of plates, the detection was carried out at 295 nm. The compact spot was obtained for Canagliflozin (R_f value 0.59 ± 0.02). The mobile phase was able to separate Canagliflozin and its degradation product formed under acid, alkaline and oxidative. Degradation of Canagliflozin was also observed in neutral and photolytic condition. Linear regression analysis data for the calibration plots showed a good linear relationship with R^2 of 0.9968 for Canagliflozin in the concentration range of 100-500 ng/band. The repeatability of measurement and repeatability of sample application of the method in terms of % RSD were found to be 0.93 % and 1.15 % respectively. The intraday and interday precision of the method in terms of % RSD was found to be 0.48-0.79% and 0.98-1.81% respectively. LOD for Canagliflozin was found to be 4.84 ng/band, while LOQ was found to be 14.67 ng/band. % Recovery of Canagliflozin was found in the range of 99.65-101.59 %. The method was successfully applied to the pharmaceutical dosage form for analysis of Canagliflozin. The assay result was found to be 99.49%.

Keywords: Canagliflozin, HPTLC, Force degradation, validation

Introduction:

Diabetes Mellitus is a metabolic disorder characterized by hyperglycaemia, glycosuria, hyperlipemia, negative nitrogen balance and sometimes ketonaemia. A prevalent pathological change is thickening of capillary basement membrane, increase in vessel wall matrix and cellular proliferation resulting in vascular complications like lumen narrowing, early atherosclerosis, sclerosis of glomerular capillaries, retinopathy, neuropathy and peripheral vascular insufficiency.



(2S,3R,4R,5S,6R)-2-[3-[[5-(4-fluorophenyl)thiophen-2-yl]methyl]-4-ethylphenyl]-6-(hydroxymethyl)oxane-3,4,5-triol

Figure 1: Structure of Canagliflozin

Sodium-glucose co-transporter 2 (SGLT2) inhibitors are a new class of diabetic medications indicated for the treatment of type 2 diabetes.

SGLT2 is a protein in humans that facilitates glucose reabsorption in the kidney. SGLT2 inhibitors block the reabsorption of glucose in the kidney, increase glucose excretion, and lower blood glucose levels.

SGLT2 is a low-affinity, high capacity glucose transporter located in the proximal tubule in the kidneys. It is responsible for 90% of glucose reabsorption. Inhibition of SGLT2 leads to a decrease in blood glucose due to the increase in renal glucose excretion.

- According to a literature survey, RP-HPLC, UV Visible spectroscopy method reported for the estimation of Canagliflozin.
- HPTLC method for the estimation of Canagliflozin in its pharmaceutical dosage form was developed and validated.

2. Material and Methods:

Instrument:

HPTLC, UV, UV Cabinet with dual wavelength UV lamp, Electronic analytical balance, Digital pH meter,

Sonicator, Controlled temperature water bath, Hot Air Oven

Material:

Aluminium plate pre-coated with silica gel 60F254 (10 × 10cm), Canagliflozin (gift samples), Toluene AR

Methanol AR, Ethyl Acetate AR, Hydrochloric acid AR, Sodium hydroxide AR, Hydrogen peroxide (6% V/V), Whatman filter paper no. 42

Preparation of Solution:

Preparation of standard stock solution of CNG:

Accurately weighed 10 mg of CNG was transferred into 10 mL volumetric flask, dissolved in few mL methanol then sonicated for 30 min and diluted up to the mark with same to get stock solution having strength 1000 µg/mL.

Preparation of working standard solution of CNG:

Aliquot of 1 mL from standard stock solution was transferred to 10 mL volumetric flask and diluted up to the mark with methanol to get solution having a strength 100 µg/mL. Further aliquot 2 ml from above solution in 10 mL volumetric flask and diluted up to the mark with methanol to get solution having a strength 20µg/mL.

Preparation of solutions for forced degradation study:

Forced degradation of CNG was carried out under acidic, alkaline, oxidative, neutral, and photolytic condition.

Acidic Degradation

Aliquot 1 mL from stock solution and transferred to 10 mL volumetric flask. was diluted up to mark with 2N HCl. Transferred the solution in the 25mL stoppered conical flask and heated for 2 hours at 80º± 2°C under water bath and cooled. Aliquot of 2 mL of above solution transferred to 10 mL volumetric flask. Neutralized with 2N NaOH and made up to 10 mL with methanol. A volume 15 µL of the resulting solution was applied in triplicate on TLC plate followed by development and scanning as described under predefined Condition.

Alkaline Degradation

Aliquot 1 mL from stock solution and transferred to 10 mL volumetric flask. was diluted up to mark with 2N NaOH. Transferred the solution in the 25mL stoppered conical flask and heated for 8 hours at 80º± 2°C under water bath and cooled. Aliquot of 2 mL of above solution transferred to 10 mL volumetric flask. Neutralized with 2N HCl and made up to 10 mL with methanol. A volume 15 µL of the resulting solution was applied in triplicate on TLC plate followed by development and scanning as described under predefined Condition.

Oxidative Degradation

Aliquot 1 mL from stock solution and transferred to 10 mL volumetric flask. was diluted up to mark with 3%H2O2. Transferred the solution in the 25mL stoppered conical flask and heated for 4 hours at 80º± 2°C under water bath and cooled. Aliquot of 2 mL of above solution transferred to 10 mL volumetric flask made up to 10 mL with methanol. A volume 15 µL of the resulting solution was applied in triplicate on TLC plate followed by development and scanning as described under predefined Condition.

Neutral Degradation:

Aliquot 1 mL from stock solution and transferred to 10 mL volumetric flask. was diluted up to mark with water. Transferred the solution in the 25mL stoppered conical flask and heated for 8 hours at 80º± 2°C under water bath and cooled. 2 mL of above solution transferred to 10 mL volumetric flask. made up to 10 mL with methanol. A volume 15 µL of the resulting solution was applied in triplicate on TLC plate followed by development and scanning as described under predefined Condition.

Photolytic Degradation:

Accurately weighed 25mg of CNG was exposed to the stability chamber for a week. Ten mg sample was transferred to 10 mL volumetric flask dissolved in few mL methanol then sonicated for 10 min and volume was made up to mark with the methanol. From the above solution, 1 mL was transferred to 10 mL volumetric flask and volume were made up to 10 mL with methanol. From the above solution, 2 mL was transferred in 10 mL volumetric flask and made up to 10 mL with methanol. A volume 15 µL of the resulting solution was applied in triplicate on TLC plate followed by development and scanning as described under predefined Condition.

Optimized Chromatographic Conditions:

The separation was performed on 10 × 10 cm aluminium backed plates pre-coated with 250 µm layer of silica gel 60F254. Samples were spotted on TLC plate 15 mm from the bottom edge using Linomat V semi-automatic spotter and analysed using the following parameters.

Preparation of calibration curve:

Aliquots of 5, 10, 15, 20 and 25 µL working standard solution of CNG (20 µg/mL) corresponding to 100-500ng/band were applied on TLC plate. Plates were developed and analysed as described under predefined condition. The calibration curve was prepared by plotting peak area of CNG against respective concentration.

Stationary phase	Aluminium plates pre-coated with Silica gel 60F254
Mobile phase	Ethyl acetate: Toluene: Methanol (2:6:2: v/v/v)
Chamber saturation time	30 min
Migration distance	75 mm
Application parameters:	
Syringe	100 µL
Application rate	100 nL/sec
Bandwidth	8 mm
Distance from the side edge of the plate	10 mm
Distance from bottom of the plate	15 mm
Scanning parameters:	
Slit dimension	6.00 × 0.40 mm, micro
Scanning speed	20 mm/sec
Detection wavelength	295 nm
Lamp	D2
Measurement mode	Absorption /Reflection

Validation of Proposed Method:

The developed method was validated as per the International Council of Harmonization (ICH) guidelines with respect to linearity, precision, specificity, Robustness, the limit of detection and limit of quantification.

Specificity

The specificity of the method was ascertained by analysing the working standard solution of CNG and pharmaceutical dosage form. Band for CNG from the sample was confirmed by comparing Rf and UV spectra with those obtained from the standard solution of CNG. Peak purity for CNG was assessed by comparing UV spectra of the corresponding band from sample acquired at 3 different positions on the band, i.e. peak start (S), peak apex (M), and peak end (E).

Linearity and Range

Linearity response of CNG was determined by analysing five independent levels of the calibration in the concentration range of 100-500 ng/band for each. A calibration curve for CNG was prepared by plotting graph of peak area against respective concentration. Correlation coefficients and regression line equations were computed.

Precision

Repeatability of measurement

15 µL of working standard solution (20 µg/mL) of CNG was applied on a TLC plate. The plate was developed as described under section 4.19. The band corresponding to CNG was scanned seven times without changing plate position and %RSD for measurement of peak area was calculated.

Repeatability of the sample application

15 µL Working standard solution (20 µg/mL) of CNG was applied on a TLC plate for seven times. The plate was developed and analysed as described under predefined Condition. Peak area of each band corresponding CNG was measured and %RSD for sample application of peak area was calculated.

Intermediate Precision

Inter-day precision (%RSD) was determined by analysing the working standard solution of CNG over the entire calibration range (100-500 ng/band) on 3 different days. The plate was developed and analysed as described under predefined condition.

Intra-day precision (%RSD) was determined by analysing the working standard solution of CNG over the entire calibration range (100-500 ng/band) for 3 times in a day. The plate was developed and analysed as described under predefined condition.

Limit of Detection and Limit of Quantification

The limits of detection of the developed method were calculated from the standard deviation of the intercepts and mean slope of the calibration curves of CNG using the equation given below:

LOD= 3.3 x SD/S

Where, SD is the standard deviation of the Y-intercepts of the seven calibration curves and S is the mean slope of the seven calibration curves.

The limits of quantitation of the developed method were calculated from the standard deviation of the intercepts and mean slope of the calibration curves of CNG using the equation given below:

LOQ = 10 x SD/S

Where, SD is the standard deviation of the Y-intercepts of the seven calibration curves and S is the mean slope of the seven calibration curves.

Accuracy

The accuracy of the developed method was determined by recovery studies at three levels (80%, 100% and 120%) by the standard addition method.

Recovery study was carried out by spiking three different known amounts (8 mg, 10 mg and 12 mg) of the standard drug to the pharmaceutical dosage form (10 mg). Three numbers of 100 mL volumetric flasks were taken. Standard CNG 8 mg, 10 mg and 12 mg were spiked in first, second, and third volumetric flasks, respectively. All three flasks were filled to about 80% with methanol, sonicated for 30 minutes and diluted up to the mark with methanol.

These solutions were filtered through Whatman filter paper individually. From each filtrate, 1 mL of each was diluted up to 10 mL with methanol individually. Each resulting solution (15 µL) was individually spotted on TLC plate. The plate was developed and analysed as described under predefined condition. From the calibration curve, the amount of CNG recovered was calculated and % recovery was determined.

Level	Amount of CNG from pre-analysed tablet powder (mg)	Spiked CNG amount CNG (mg)	Concentration of CNG solution (µg/mL)
80%	10	8	18
100%	10	10	20
120%	10	12	22

Robustness

Robustness (%RSD) was determined by analysing the working standard solution of CNG over the entire calibration range on the changed wavelength (± 2nm), Scanning speed (10,20,40 mm/sec) and changed saturation time (±5min). The plate was developed and analysed as described under predefined condition.

Assay of pharmaceutical dosage form

The pharmaceutical dosage form was analysed using the developed method. Accurately weighed from formulation equivalent to 10 mg CNG was transferred to 100 mL volumetric flask, dissolved in few mL methanol then sonicated for 30 min and diluted up to the mark with methanol and filtered through Whatman filter. Aliquot 2 mL from the filtrate was transferred into 10 mL volumetric flask and diluted up to the mark with methanol. A volume 15 µL of the resulting solution was applied in triplicate on TLC plate followed by development and scanning as described under predefined condition. The amount of CNG present in the sample solution was determined by fitting area values of the corresponding peak into the equation representing the calibration curve of CNG and % labelled claim of the drug was computed.

Result and Discussion:

Preparation of Calibration Curve

A calibration curve was prepared in the range of 100-500 ng/band for CNG. The peak area of CNG increases linearly with concentration. Data of calibration curve is shown in table. Calibration curve of CNG is depicted in figure 2.

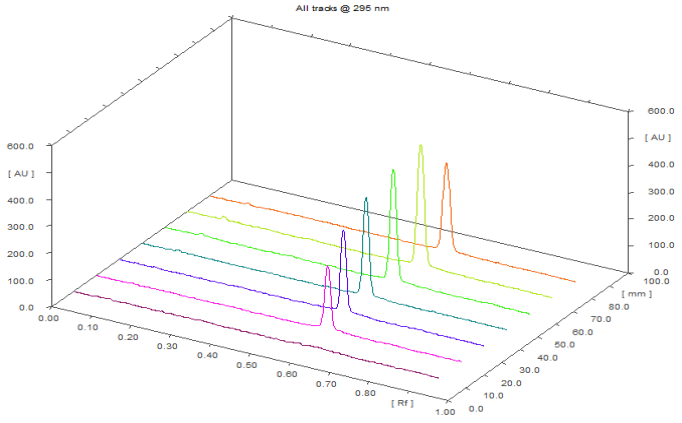


Figure 2: 3D Chromatogram of calibration curve of CNG (100-500 ng/band)

Concentration (ng/band)	Peak area of CNG
100	3315.4
200	4228.7
300	5077.9
400	6135.9
500	7174.4

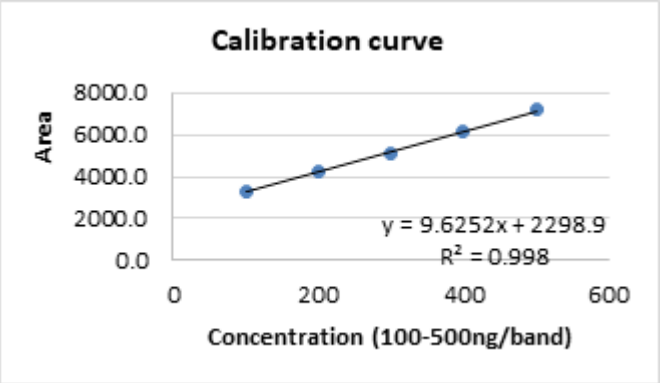


Figure 3: Calibration curve for CNG (100-500 ng/band)

Forced degradation study

Acidic Degradation

Degradation of CNG was observed in acidic condition. Peak area for CNG has decreased an additional peak of degradation product was found in the chromatogram (Figure 5.12). CNG was found to be degraded about 34.29 % after heating with 2 N HCl at 80º± 2°C for 2 hours.

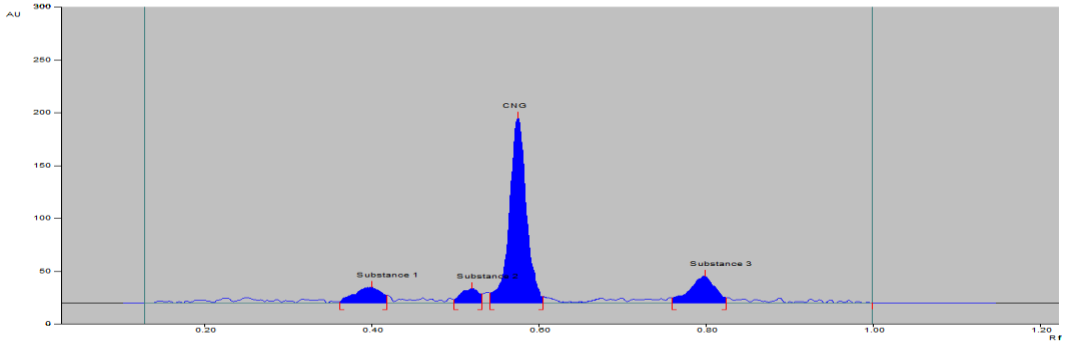


Figure 4: Densitogram of acid degradation study for CNG; condition: 2 N HCl at 80º± 2ºC for 2 hours; CNG (Rf: 0.59),degraded product(Rf:0.40,0.52,0.80)

Alkaline Degradation

Degradation of CNG was observed in alkaline condition. Peak area for CNG has decreased an additional peak of degradation product was found in the chromatogram (Figure 5.13). CNG was found to be degraded about 16.25 % with 2 N NaOH at 80º± 2°C for 8 hours.

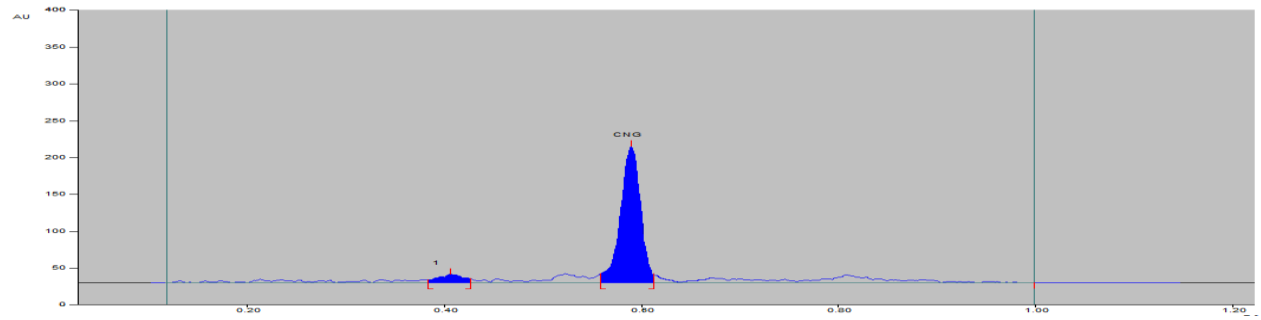


Figure 5: Densitogram of alkaline degradation study for CNG; condition: 2N NaOH at 80⁰± 2°C for 8 hours; CNG (Rf : 0.59), degraded product(Rf:0.40)

Neutral Degradation

Degradation of CNG was observed in the neutral condition. Peak area for CNG has decreased an additional peak of degradation product was not found in the chromatogram (figure 5.14). CNG was found to be degraded about 8.15% after heating with water at 80^0 ± 2°C for 8 hours.

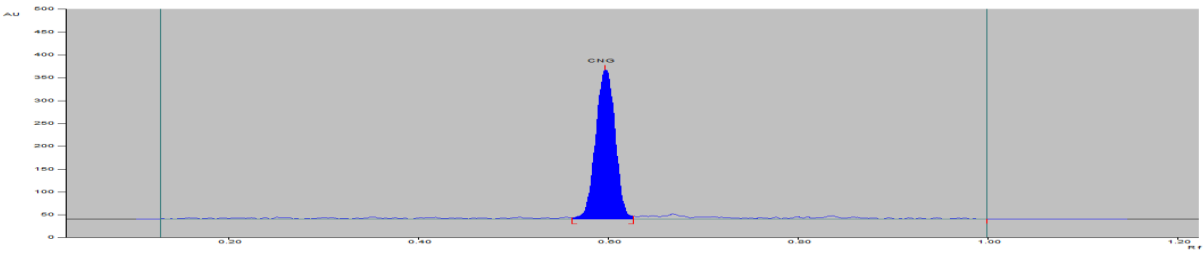


Figure 6: Densitogram of Neutral degradation study for CNG; condition: at 80⁰± 2°C for 8 hours; CNG (Rf : 0.59)

Oxidative degradation

Degradation of CNG was observed in oxidative condition. Peak area for CNG has decreased an additional peak of degradation product was found in the chromatogram (Figure 5.15). CNG was found to be degraded about 23.16 % after heating with 3% H2O2 at 80⁰± 2°C for 8hours.

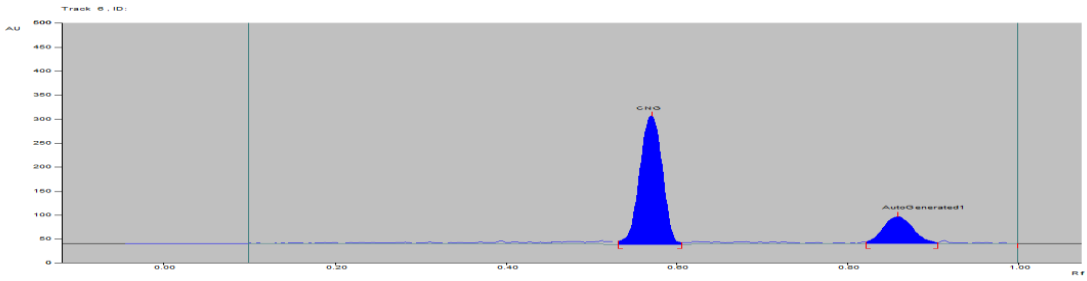


Figure 7: Densitogram of oxidative degradation study for CNG; condition: 3 % H2O2 at 80⁰± 2°C for 4 hours; CNG (Rf: 0.59),degraded product (Rf : 0.83)

Photolytic degradation

Degradation of CNG was observed in Photolytic condition. Peak area for CNG has decreased and an additional peak of degradation product was not found in the chromatogram (Figure 5.16). CNG was found to be degraded about 1.54 % in photo-stability chamber at 25^0 ± 2°C for 1 week.

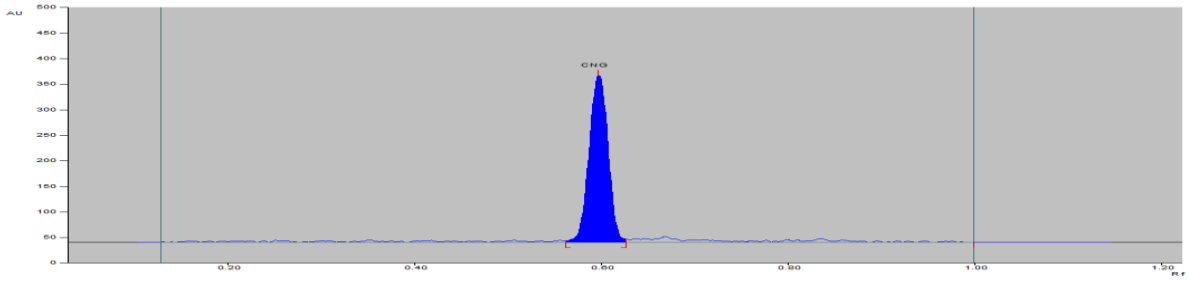


Figure 8: Densitogram of photolytic degradation study for CNG; condition: drug is exposed to Stability chamber for 1 week; CNG (Rf: 0.59)

Table 1 Summary of forced degradation study of CNG

No.	Stress type	Stress conditions	%Degradation
1	Acid hydrolysis	2N HCl at 80°C for 2 hours	34.30
2	Alkaline hydrolysis	2N NaOH at 80°C for 8 hours	16.25
3	Oxidative degradation	3% H2O2 at 80°C for 4 hours	23.07
4	Neutral hydrolysis	80°C for 8 hours	8.37
5	Photolytic degradation	Stability chamber for 1 week 25±2°C	1.54

Method Validation

Specificity

Comparison of chromatograms of CNG from standard and that from pharmaceutical dosage form showed identical Rf values of CNG 0.59 ± 0.02 (Figure 5.18 and Figure 5.19). Apart from Rf values, the UV spectrum of the individual band from pharmaceutical dosage form was also correlated with the spectrum of standard CNG. Comparison of the spectra scanned at peak start(s), peak apex (m) and peak end (e) positions of individual bands of standard showed a high degree of correlation (Figure 5.19), confirmed the purity of the corresponding bands. Peak purity data of CNG is depicted in Table 5.16. Spectra of pharmaceutical dosage form (Figure 5.20), confirmed the purity of the corresponding bands.Spectra of pharmaceutical dosage form and standard were superimposable (Figure 5.21) which confirms identity CNG from the pharmaceutical dosage form.

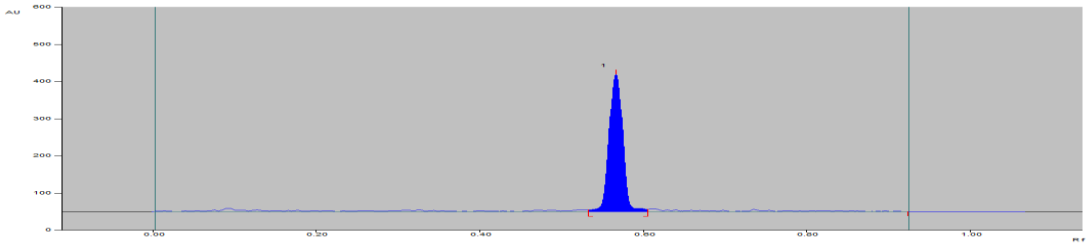


Figure 9: Densitogram of standard CNG (300ng/band); CNG (Rf: 0.59 ± 0.02)

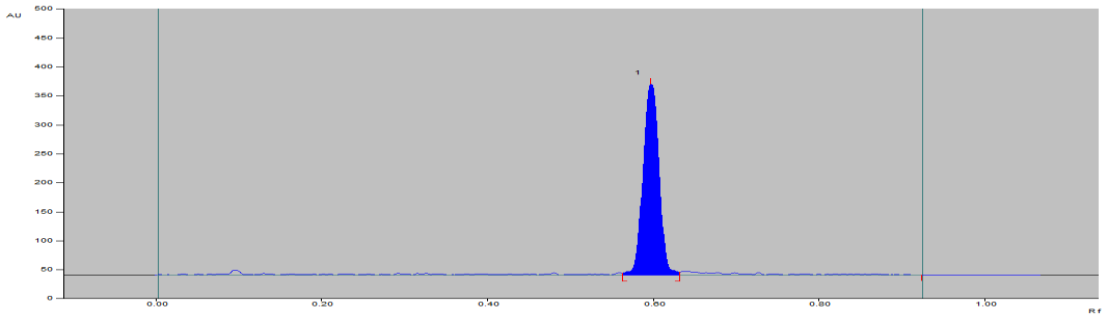


Figure 10: Densitogram of CNG from pharmaceutical dosage form (300ng/band); CNG (Rf: 0.59 ± 0.02)

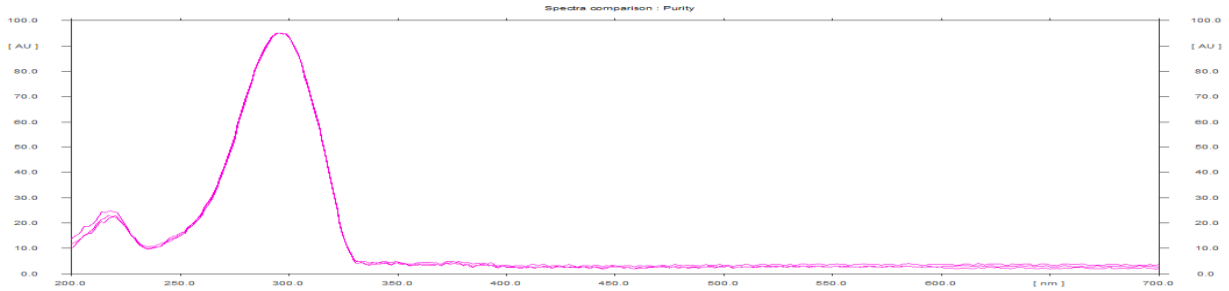


Figure 11: Peak purity spectra of standard CNG [at peak start (s), peak apex (m) and peak end (e)]

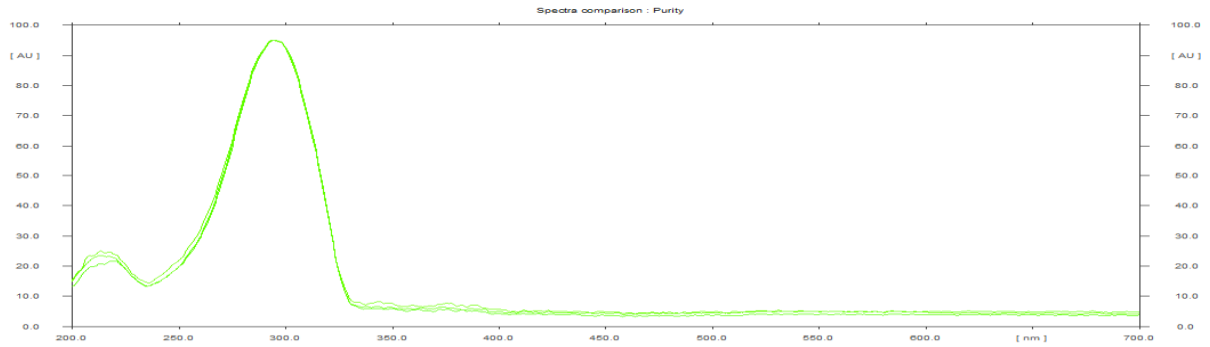


Figure 12: Peak purity spectra of CNG from pharmaceutical dosage form[at peak start (s), peak apex (m) and peak end (e)]

Table:2 Data of Peak Purity

Purity Drug	R(s,m)	R(m,e)
CNG	0.9997	0.9996
Pharmaceutical dosage form	0.9997	0.9998

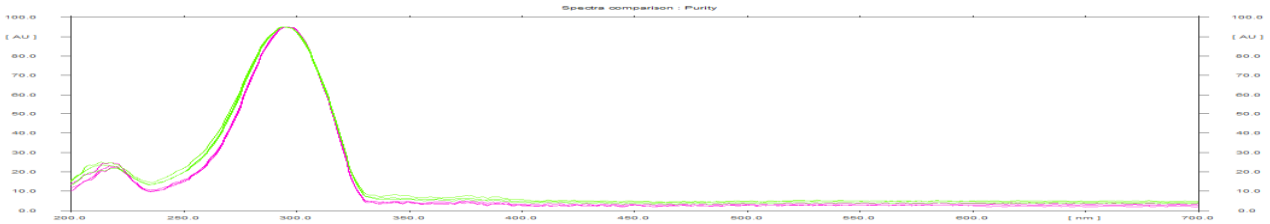


Figure 13: Overlain spectra of CNG standard and pharmaceutical dosage form [at peak apex (m)]

Linearity and Range

Representative calibration curve of CNG was obtained by plotting the mean peak area of CNG against concentration over the range 100-500 ng/band (n=7). It was found to be linear in the above-mentioned range with a coefficient of regression 0.996. The % RSD for each level of CNG was found to be in the range of 0.51-0.71%. The average linear regressed equation for the calibration curve was Y= 9.7145x + 2233.7 (Figure 5.22). Linearity data is depicted in Table 5.17 and summary data depicted in Table 5.28**.**

Table 3: Calibration data for CNG

Concentration (ng/band)	**Peak area (Mean ± SD) (n=5)**	**%RSD**
100	3286.17±23.29	0.71
200	4156.86±25.13	0.60
300	5040.00±34.61	0.69
400	6070.31±37.46	0.62
500	7186.67±36.33	0.51

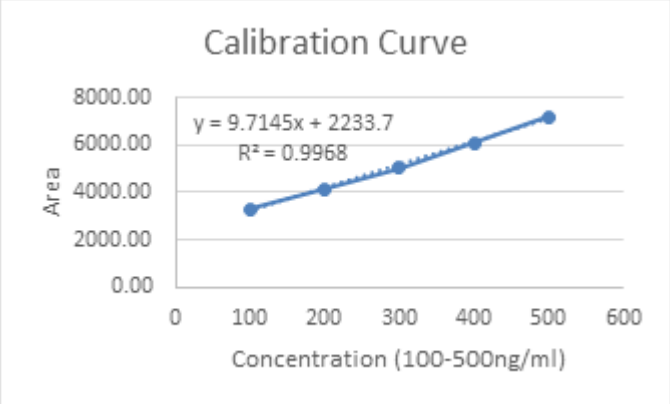


Figure 14: Calibration curve of CNG (100-500ng/band)

Table 4: Summary of linearity data

Parameters	**Results**
Linearity range	100-500 ng/band
Slope	9.7145
Intercept	2233.7
Regression coefficient (R^2)	0.9968

Precision

Repeatability of measurement

The precision of instrument was checked by scanning same spot (working standard solution, 15 µL) seven times without changing plate position. % RSD of measurement (15 µL) of peak area was found to be 0.93% for CNG. These results indicate that the method is precise for measurement of CNG. Data for repeatability of measurement is depicted in Table 5.19.

Table 5: Repeatability data of measurement for estimation of CNG

CONCENTRATION (NG/BAND)	PEAK AREA (MEAN ± SD) (N=7)	%RSD
300	5061.6 ± 47.16	0.93

Repeatability of the sample application

Repeatability of sample application (15 µL) for CNG was found to be 1.15 %, which ensures the precision of spotter device. Data is depicted in Table 5.20 and 3D chromatogram of repeatability of sample application of CNG has been depicted in Figure 5.23.

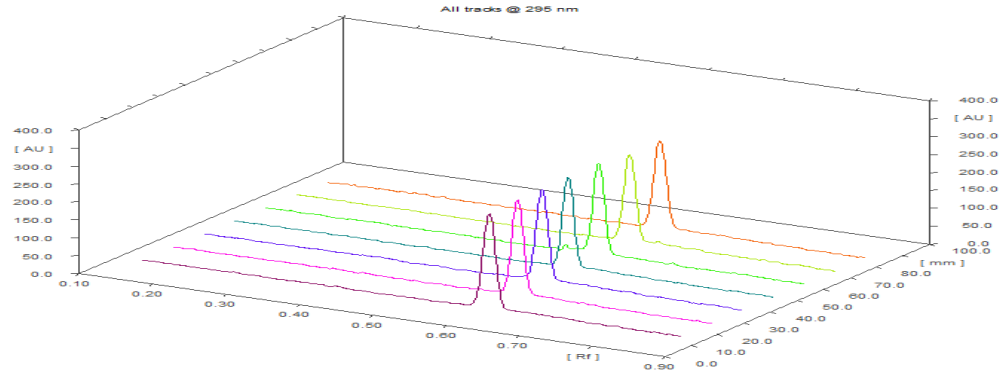


Figure 15: 3D Chromatogram of repeatability of sample application of CNG (300 ng/band)

Table 6: Repeatability data of sample application for estimation of CNG

Concentration (ng/band)	***Peak area (Mean ± SD) (n=7)***	***%RSD***
300	5114.3 ± 59.04	1.15

Intermediate Precision

The % RSD for intra-day precision of CNG was found to be in the range of 0.48-0.79%, while %RSD for inter-day precision of CNG was found to be 0.92-1.81%. These results indicate that the method is precise for measurement of CNG. Data for intra-day and inter-day precision is depicted in Table 5.21.

Table 7: Data for Intermediate precision for estimation of CNG

Concentration (ng/band)	**Intra-day precision**		**Inter-day precision**	
	Peak area (Mean ± SD) (n=3)	**% RSD**	**Peak area (Mean ± SD)(n=3)**	**% RSD**
100	3308.03±15.80	0.48	3316.70±32.89	0.99
200	4234.13±32.59	0.77	4108.53±74.36	1.81
300	5052.43±39.86	0.79	5073.73±65.85	1.30
400	6100.63±36.45	0.60	6047.37±55.60	0.92
500	7208.03±34.86	0.48	7187.03±70.09	0.98

Limit of detection and Limit of quantitation

LOD and LOQ were determined from the mean slope and standard deviation of the y-intercept of seven calibration curves. LOD for CNG was found to be 4.84 ng/band, while LOQ for CNG was found to be 14.67 ng/band. Data are depicted in Table 5.22.

Table 8: LOD and LOQ Data for CNG

Parameters	**Results**
The standard deviation of the Y-intercepts of Calibration curves (n=7)	14.25
The mean slope of calibration curves (n=7)	9.71
LOD = 3.3 × (SD/Slope) (ng/band)	4.84
LOQ = 10 × (SD/Slope) (ng/band)	14.67

Accuracy

Accuracy was determined in terms of recovery study and the recovery are done at three levels i.e. 80 %, 100 % and 120 %. The % recovery of CNG was found to be in the range of 99.65-101.29 %. These results indicate that the method is accurate in the measurement of CNG. The data for the accuracy of the method for CNG is depicted in Table 5.23.

Table 9: Recovery data of CNG

Spiked level (%)	**Amount of CNG from pre-analyzed tablet powder (mg)**	**Spiked CNG amount (mg)**	**Average peak area (n=3) ± SD**	**Amount found (mg)**	**Recovery (%)**
80	10	8	4717.33±11.08	8.05	100.59
100	10	10	4981.168±5.00	9.96	99.65
120	10	12	5282.63±20.75	12.16	101.29

Robustness

The %RSD for different wavelength, scanning speed and different saturation time of CNG was found to be in the range of 0.15-1.60 %, 0.49-1.0 and 0.16-1.08 % respectively. These results indicate that the method is robust for measurement of CNG. The data for different wavelength, scanning speed and different chamber saturation time for CNG is depicted in Table 5.24,5:25 and 5;26.

Table 10: Data for robustness(change in wavelength)

Concentration (ng/band)	Wavelength			AVG	SD	RSD
	293	**295**	**297**			
100	3296.3	3223.6	3196.1	3238.7	51.75	1.60
200	4006.2	3972.1	3962.1	3980.1	23.13	0.58
300	4789.7	4844.0	4766.2	4800.0	39.92	0.83
400	5841.0	5853.3	5784.9	5826.4	36.48	0.63
500	6921.2	6914.7	6934.6	6923.5	10.17	0.15

Table 11: Data for robustness (Scanning Speed)

Concentration (ng/band)	Scanning Speed			AVG	SD	RSD
	10	**20**	**40**			
100	3230.2	3223.6	3282.7	3245.5	32.37	1.00
200	3968.0	3972.1	3916.1	3952.1	31.21	0.79
300	4791.1	4844.0	4874.9	4836.7	42.36	0.88
400	5825.3	5853.3	5802.4	5827.0	25.51	0.44
500	6912.7	6914.7	6855.1	6894.2	33.85	0.49

Table 12: Data for Robustness (Saturation Time)

Concentration (ng/band)	Saturation Time			AVG	SD	RSD
	15min	30min	45 min			
100	3274.2	3229.8	3223.6	3242.5	27.59	0.85
200	3913.4	3928.7	3972.1	3938.1	30.43	0.77
300	4741.5	4804.4	4844.0	4796.6	51.70	1.08
400	5844.7	5820.2	5853.3	5839.4	17.19	0.29
500	6918.3	6928.4	6906.9	6917.9	10.76	0.16

Validation summary

Summary of all validation parameters has been presented in Table 5.27.

Table 13: Summary of Validation Parameter

Sr. No.	Parameters	Results
1	Linearity Range	100-500 ng/band
2	Regression equation	y = 9.7145x + 2233.7
3	Regression coefficient(R2)	0.9968
4	Precision (%RSD)	
	Repeatability of measurement(n=7))	0.93
	Repeatability of sample application(n=7)	1.15
	Intermediate precision	
	Intra- day precision(n=3)	0.48-0.79
	Inter-day precision(n=3)	0.98-1.81
5	Limit of detection(LOD)	4.84 ng/band
6	Limit of quantification(LOQ)	14.64 ng/band
7	Accuracy(n=3)	99.65-101.59%
8	Robustness	Robust

Analysis of Pharmaceutical dosage form

Applicability of the proposed method was tested by analysing pharmaceutical dosage form. The percentage of CNG in the pharmaceutical dosage form was calculated from the calibration curve of CNG. The assay value for the pharmaceutical dosage form of CNG was

99.49%. This result is within the range of precise limit. Assay result of the pharmaceutical dosage form of CNG is depicted in table 5.28.

Table 14: Data for assay

Labelled claim (mg)	Average peak area (n=3) ± SD	Amount of drug found (mg)	Assay (%)
100	4972.16±9.18	99.49	99.49

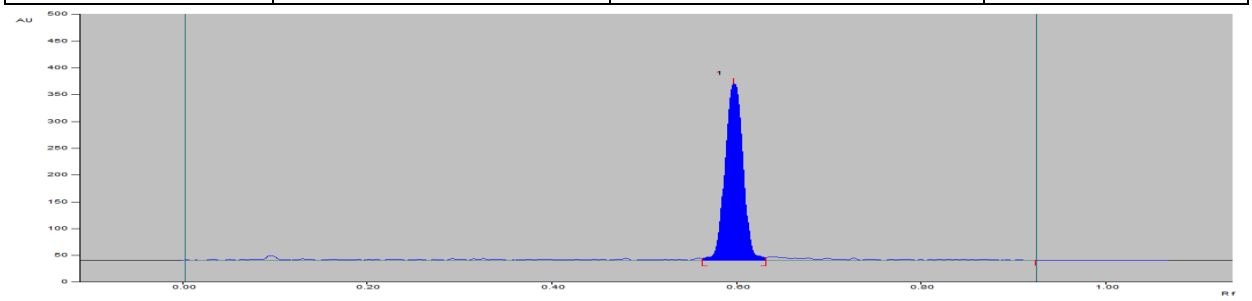


Figure 16: Densitogram of Pharmaceutical dosage form

Conclusion:

Stability indicating HPTLC method was developed and validated for the Estimation of CNG in the tablet dosage form. The proposed method was found to be specific, accurate, precise and sensitive. Forced degradation study was conducted by applying different stress conditions i.e. acidic, alkaline, oxidation, neutral and photolytic. The developed method was applied for the assay of pharmaceutical dosage forms and results were found to be in good agreement with the extract amount. The proposed method can be applied for the routine analysis of tablet dosage form.

References:

1. Tripathi KD(2008). Essential of Medical Pharmacology. Jaypee Brothers Medical Publishers, New Delhi. 6th ed. :254-66
2. Mechanism of Canagliflozin. November 2018
3. www.diabetesincontrolcom/sglt2-inhibitors-a-new-class-ofdiabetes-medications.
4. Canagliflozin drug information in Drugbank, November 2018
5. https://wwwdrugbankca/drugs/DB08907
6. Canagliflozin drug information in pubchem, November 2018 https://pubchemncbinlmnihgov/compound/Canagliflozin#section=Other-Identifierss
7. Shethi PD(1996). High Performance Thin Layer Chromatography-Quantitative Analysis of Pharmaceutical Formulations. 1st ed. CBS Publishers, New Delhi; pp. 1-68.
8. Ranger B(1993) Quantitative Planer Chromatography as a tool in Pharmaceutical Analysis. Journal of AOAC. 1993;76(20):7-13.
9. Jaenchen D(1997), Frank AS, Handbook of Instrumental Techniques for Analytical Chemistry. 1st ed. New Jersey: Prentice Hall; pp. 221-235.
10. Guideline. IHT. Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Council of Harmonization, Geneva: Switzerland. 2005.
11. Industry. Gf. Analytical Procedures and Methods Validation: Chemistry, Manufacturing,and Controls Documentation. US Department of Health and Human Services Food and Drug Administration. 2000:4.

SMALL MOLECULES BASED α-HELIX MIMETIC FOR PROTEIN SURFACE RECOGNITION IN DRUG DISCOVERY

Krishnananda Samanta
Department of Chemistry,
Balurghat College, University of Gour-Banga, 733101, West Bengal, India
Corresponding author E-mail: krishnanandasamanta5@gmail.com

Abstract:

Mimicking the secondary structure of a protein by rational design of synthetic molecules to inhibit the protein-protein interactions is important therapeutic approach in the field of drug discovery. Here we have described small molecules based α-helical mimetic, that efficiently bind on protein surface and inhibit protein-protein interaction.

Keywords: α-helix mimetic, protein-protein interaction, drug discovery

Introduction:

Design of synthetic molecules that can effectively bind protein surface are very fascinating and modern biomedical field as it is the principal route to discovery of new medicine that prevents several fatal diseases (Peczuh *et al*., 2000). There are two categories of interaction sites of proteins: (a) inside or interior interaction protein site and (b) surface interaction protein site. In most cases, enzyme active sites are found at the interior part of proteins. The success of functionalized small molecules that inhibit enzyme activity is due to convergent nature of enzyme active functional group. However, protein surface functional group is divergent in nature. As it is known the unique composition of charged, hydrophobic and hydrophilic domains on every protein surface, synthetic molecules that match the electrostatic features and topology of the targets protein surface, might be expected to bind target protein and prevent protein-protein interaction (Vallone *et al*., 1998). Therefore, tight binding requires the involvement of large surface areas and multiple functional points.

The understanding and manipulation of protein-protein interaction are of growing interest due to their critical role in the cellular function/organelle structure, immune response, protein enzyme inhibitors, signal transduction, and apoptosis. Rational design of synthetic molecules toward the recognition of protein surfaces may provide better insights into exactly how proteins interact with one another and is an alternative way of disease therapy. Identification of "hot spots" on protein surface is an important development of protein structure field (Clackson *et al*., 1995). The functional groups or residues that comprise the hot spot, contribute significantly to the stability of the protein-protein complex formation.

Designed synthetic molecules that disrupt protein/protein interactions through specific recognition sites must satisfy two complementary criteria: (1) the recognition of protein surfaces in ways that agonize a biological response, mediate some protein dimerization, or stabilize the native oligomerization state of the protein, (2)targeting a protein surface would be to selectively bind half of a dimeric interaction in a way that would sterically block association with the natural protein partner (Peterson *et al*., 1998).

The effort of this chapter is to present current strategies for binding protein surfaces with emphasis on the use of designed molecules. The principles of molecular recognition learned from these model systems are putting the foundation for progress in the field of protein surface recognition.

The design of low-molecular weight ligands with a desired helical propensity that disrupts protein-protein interactions is a challenging area in medicinal chemistry due to the involvement of a large interfacial surface area (Cochran 2001). Over 30% of protein secondary structure is helical in nature, making it the most abundant secondary structural motif. It stands to reason therefore that a significant population of PPIs (protein-protein interactions) should involve α-helices, representing a generic template for inhibitor design (Azzarito *et al*., 2013).

NK3 protein surface:

The pioneering work of Horwell *et al*. showed that conformationally constrained, nonpeptide templates (l,l,6-trisubstituted indanes) (Fig. 1) allow the incorporation of two adjacent amino acid side chains, plus a third binding group in an orientation similar to that found in alpha-helices. Several racemic and homochiral Phe-Phe and Trp-Phe mimetics were prepared. Their binding affinity was evaluated against tachykinin receptor through binding assays. Many of them showed strong binding affinity to the NK, and/or NK3 receptor. The X-ray crystallography of the homochiral indanes, *(1R)-N-((S)-l-hydroxymethylbenzyl)-l,6-dibenzylindan-l-carboxamide* was analyzed and was found to be in an α-helix conformation (Horwell *et al*., 1996).

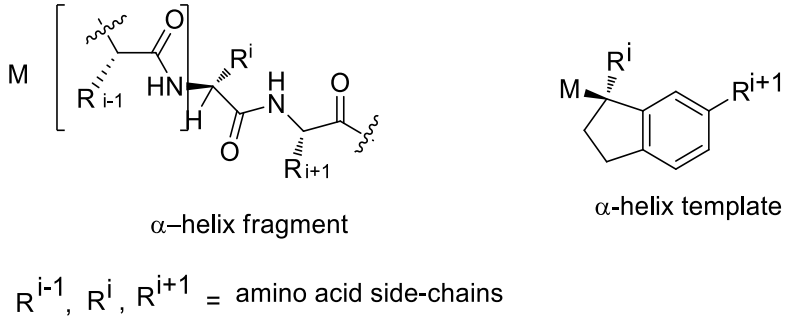


Figure 1: The indane template (right) mimicking an α-helix.

Bak/Bcl-xL protein surface:

Kutzki *et al.* (2002*)* designed a potent α-helix mimicry based on the crystal and solution structures of Bak/Bcl-xL complex (Fig. 2). Many types of cancer are linked to overexpress of Bcl-xL which protects transformed cells from cell death, leading to uncontrolled cell growth. A series of terphenyl molecules (**1**-**5)** (Fig. 3) have been designed and synthesized, containing alkyl or aryl substituents on the three ortho positions to mimic the key hydrophobic substituents (*i*, *i* + 3, and *i* + 7) on the helical exterior of Bak or Bad and carboxylic acid substituents on either end to mimic the additional ion pair. A fluorescence polarization (FP) assay showed that the terphenyl molecule (**4**) shows the strongest binding to Bcl-xL with a K_D value of 114 nM. The compounds (**1** and **3**) showed lower affinity (K_D) 2.09 and 1.89 μM, respectively), indicating the importance of hydrophobic interactions for recognition in Bcl-xL. When position of the naphthaline substituent, was changed in **5**, leads to a significant drop in binding affinity (K_D) 2.70 μM), suggesting an effective shape complementarity for **4**, as in the natural peptide.

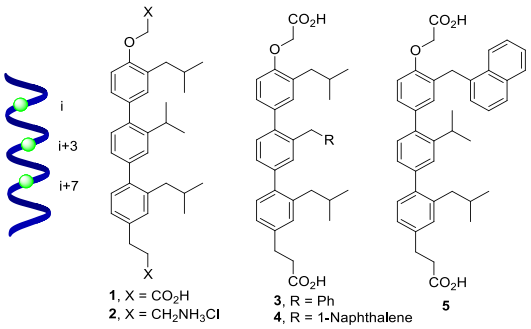


Figure 3: List of compounds tested in the FP assay

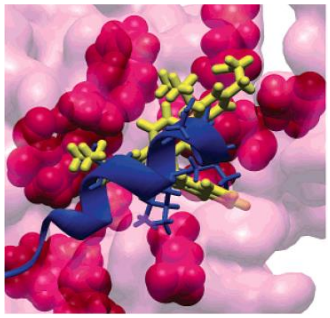
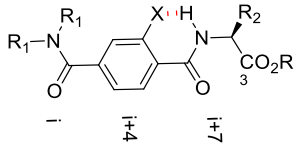


Figure 2: Overlay of peptide and postulated binding location for 4. (Kutzki *et al.*, 2002)

Yin *et al.* (2005) reported a series of terephthalamide scaffold-based helical mimetics (Fig. 4) that mimic the α-helical region of the Bak peptide. Among the library of terephthalamide derivatives, compounds **6** (K_i = 0.78 ± 0.07) and **7**(K_i = 1.85 ± 0.32 μM), showed favorable *in vitro* activities in disrupting the Bcl-xL/Bak BH3 domain complex (Fig. 4). NMR experiments and computational docking simulations proposed that the synthetic inhibitors bind to the cleft of BH3 domain of the Bak peptide on the surface of Bcl-xL. The intramolecular hydrogen bond between the amide -NH and the alkoxy oxygen atom play an important role to induce conformational constraint in the molecule that resulted in the preferred orientation of the 2-isopropoxy group and the upper isobutyl side chain on the same side of the terephthalamide.



6, R_1 = -*i*Pr, X = -OiPr, R_2 = -*i*Bu, R_3 = -Me

7, R_1 =- *i*Pr, X = -OMe, R_2 = -(S)-*i*Bu, R_3 = -Me

Figure 4: Generic structure of terephthalamide helical mimetic

Rodriguez *et al.* (2009*)* reported a biphenyl 4,4'-dicarboxamide scaffold-based structure designed to mimic the i, i+4, i+7, and i+11 residues of a Bcl-xL/Bak protein–helix interface (Fig. 5). This scaffold combines the hydrophobic core of the oligophenyl series and the synthetically accessible carboxamide groups of the terephthalamides. An energy-minimized structure of biphenyl (in which R_1=R_2=R_3=R_4=Me) showed good overlap with the i, i+4, i+7, and i+11 residue side chains of an α-helix with an RMSD value of 1.368. The results display (Table 1) that the biphenyl derivatives inhibit the Bcl-xL/Bak interaction. The best biphenyl, **10**, was found to have a K_i value of 1.8 µm by FP and a K_d of 7.1 µm, as determined by isothermal *titration* calorimetry (ITC).

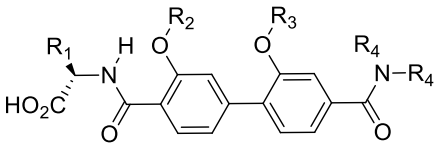


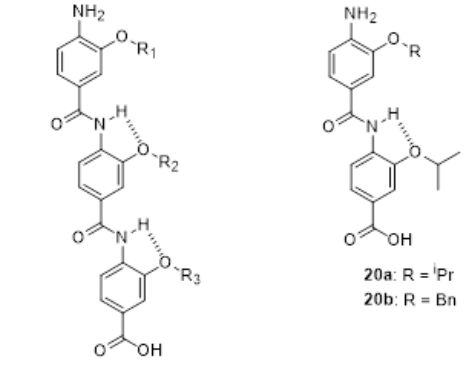
Figure 5: List of biphenyl-based α-helical mimetics (8-18) and their FP assay results

Table 1: Competition fluorescence polarization assay results for biphenyls 8-18.

Compound	R_1	R_2	R_3	R_4	K_i[µM]
8	Me	*i*Pr	Ph	*i*Pr	180±55
9	*i*Bu	*i*Pr	Ph	*i*Pr	36±6.3
10	Me	2-naphthyl	*i*Pr	*i*Pr	37±7.0
11	*i*Bu	2-naphthyl	*i*Pr	*i*Pr	17±6.1
12	Me	*i*Pr	2-naphthyl	*i*Pr	29±6.2
13	*i*Bu	*i*Pr	2-naphthyl	*i*Pr	2.1±0.57
14	Me	2-naphthyl	Ph	*i*Pr	8.6±0.67
15	*i*Bu	2-naphthyl	Ph	*i*Pr	9.6±0.57
16	Bn	*i*Pr	2-naphthyl	*i*Pr	>500
17	*i*Bu	*i*Pr	1-naphthyl	*i*Br	1.8±0.63
18	*i*Bu	*i*Pr	1-naphthyl	*i*Pr	2.3±0.57

p53–hDM2 protein surface:

Wilson *et al*. (2009) described oligobenzamide scaffold-based α-helix proteomimetic inhibitors of the p53–hDM2 protein–protein interaction (Fig. 6**)**. P53 is the major tumor suppressor and its activity is regulated by binding to hDM2. Over-expression of hDM2 inhibited p53 function and thus, PPI is an important therapeutic target for cancer chemotherapy. Three key hydrophobic residues Phe19, Trp23, and Leu26 from p53 have involved the interaction between p53 and hDM2 binding in a helical conformation to a hydrophobic cleft on hDM2 (Fig. 7). A series of oligobenzamide compounds were synthesized, considering the mimic of side chains key residues at I, I + 4 and I + 7 of the p53 helix and affinity was tested in a fluorescence anisotropy assay. All compounds (**19a-f**) were found to inhibit the interaction with low µM activity. The best inhibitor was **19e** with IC_{50} of 1.0 µM was determined. Compounds **20a and 20b** are negative controls.



19a: $R_1 = R_2 = R_3 =$ *iso*-propyl
19b: $R_1 =$ *iso*-propyl, R_2 = Bn, $R_3 = n$-propyl
19c: R_1 = 1-napth, R_2 = Bn, $R_3 =$ *iso*-propyl
19d: R_1 = 2-napth, R_2 = Bn, $R_3 =$ *iso*-propyl
19e: R_1 = Bn, R_2 = 2-napth, $R_3 =$ *iso*-propyl
19f: R_1 = Bn, R_2 = Bn, $R_3 =$ *iso*-propyl

Figure 6: Oligobenzamide scaffold based-helix proteomimetics

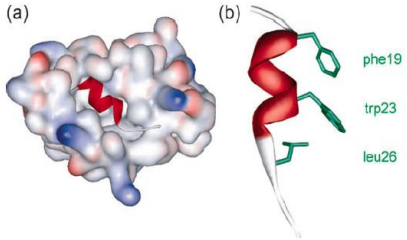


Figure 7: (a) Crystal structure of hDM2 in complex with a p53 peptide (PDB ID: 1YCR) and (b) the p53 peptide showing side chains key for binding. (Wilson *et al*., 2009)

The same group introduced a 'hybrid' α-helix mimetics in which the backbone was varied by substitution of the middle aryl unit with an α-amino acid to mimic the I, I + 4 and I + 7 side-chains of the p53 α-helix which forms a PPI with hDM2 (Fig. 8). The Phe19, Trp23, and Leu26 of p53 are important residues present in the hot-spot. The designed foldamers should mimic this hot spot to inhibit protein–protein interaction of hDM2/ p53. All mimetics were tested in fluorescence anisotropy (FA) competition assay. Hybrid 2**1** displayed low micromolar affinity (IC_{50} of 11.9 ±0.6 mM) and all controls 2**2–25** were found inactive. The inactivity of all controls 2**2-25** indicated that the 'top', and 'bottom' units and hydrophobic side chains play a significant role in binding. Most significantly, mimetic 2**1** acted as a selective inhibitor for p53/hDM2 against four other targets. The selectivity for p53/hDM2 inhibition versus Mcl-1/NOXA-B inhibition was realized upon changing the stereochemistry of the side chain L-Phe residue with D-Phe of the α-helix mimetic (Azzarito *et al*., 2015).

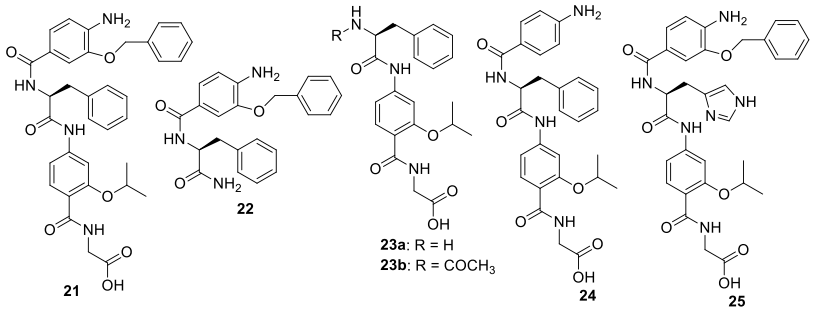


Figure 8: List of 'hybrid' α-helix mimetics

Lee *et al*. (2011) reported the design of a novel pyrrolopyrimidine based α-helix mimetics with increased conformational rigidity and identified the most potent, dual inhibitors of MDMX- and MDM2-p53 interactions through high-throughput screening (Fig. 9**)**. To find out such inhibitors, they constructed a 900-member library of compound **26** following a facile solid-phase divergent synthetic route. The binding affinities of the selected compounds (**26a**, **26b**) were tested using a fluorescence polarization-based assay. The compounds **26a** and **26b** effectively inhibited the p53-MDMX binding with K_i = 0.62 and 0.45 μM, respectively, which were comparable with that of a 15-mer p53peptide (K_i = 0.8 μM) and also were found to inhibit the p53-MDM2 interaction as well, with K_i = 0.62 and 0.84 μM, respectively, similar to the binding affinities for MDMX. Computational modeling showed that scaffold **26** could mimic MDMX-bound p53 peptide (Fig. 10).

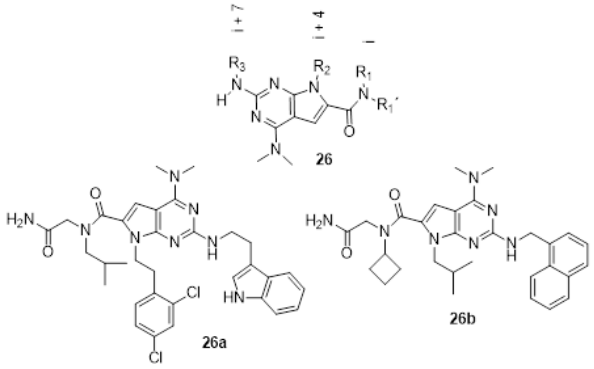


Figure 9: Pyrrolopyrimidine-Based α-helix mimetics

Figure 10: Overlay of compound 26 (R1, R2, R3 =CH3) and an MDMX-boundp53 peptide (Lee *et al*.,2011)

(HIF1α)/ p300 protein surface

Arora *et al*. (2014) described the design, synthesis, biochemical, and in vivo evaluation of an oxopiperazine helix mimic (OHM) that captures the topography of α-helical domain at the interface of hypoxia-inducible factor 1α and p300 to develop inhibitors of hypoxia-inducible signaling (Fig.11). It regulates the transcription of key genes, whose expression associated with metastasis, angiogenesis, and altered energy metabolism in cancer. The designed oxopiperazine helix mimics (**OHM 27**) (Fig. 12) bound to the hotspot of the target protein with high affinity and inhibited the interactions of hypoxia-inducible factor 1α (HIF1α) with coactivator p300/CBP. As a result, downregulated the expression of certain genes and reduced tumor burden in mouse xenograft models. **OHM 27** targets CH1 with an affinity of K_d = (5.3 ± 1.4) × 10^{-7} M and **OHM 28**, which comprises the two critical leucine residues but lacks Gln824, bound with a slightly reduced affinity K_d = (6.2 ± 1.1) ×10^{-7} M. The two negative controls **OHMs 29** and **30** showed very weak affinities for p300–CH1, with K_d values of >>1.0 × 10^{-5} M in each case.

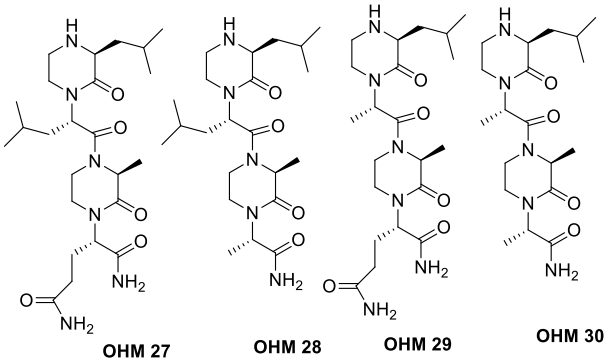


Figure 11: Synthesis of topographical HIF1α mimetic as modulators of hypoxia-inducible gene expression.

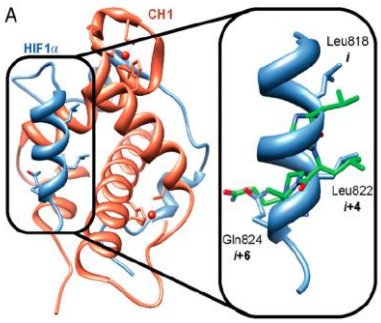


Figure 12: An overlay of the HIF1α helix spanning residues 816–824 (blue) and OHM 27 (green). (Arora *et al*., 2014)

Burslem *et al*. (2014) designed and synthesized the first examples of aromatic oligoamide helix mimetic inhibitors (Fig. 13) of the HIF-1a–p300 protein–protein interaction. The hypoxia-inducible factor (HIF), plays a central role in the cellular response to hypoxia that results in the expression of multiple genes (e.g., VEGF) which participate in angiogenesis, various metabolic processes, and cell proliferation and survival. The designed compounds are intended to mimic the key functionalities at the i, i+4, and i+7 positions and spatial orientation of the C-terminal helix (helix 3) of HIF-1a. IC_{50} values of the mimetics are summarised in Table 2. Compounds **31** and **32** were designed based on helix 3which was shown to be inactive in the fluorescence anisotropy assay. Compound **34** was the most potent inhibitor, but it is less potent than HIF-1α.

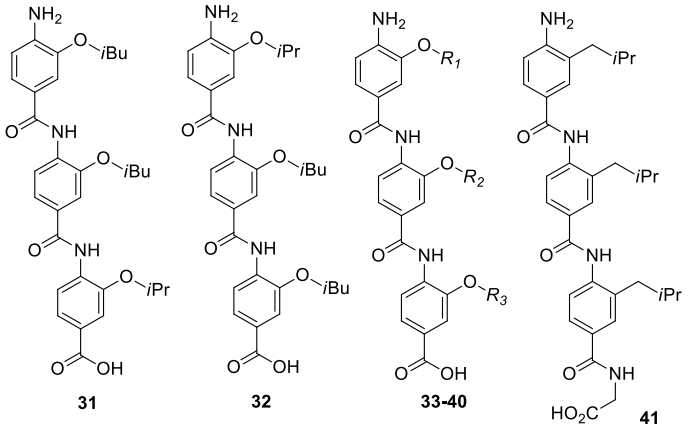


Figure 13: List of aromatic oligoamide helix mimetic inhibitors and their IC_{50} values

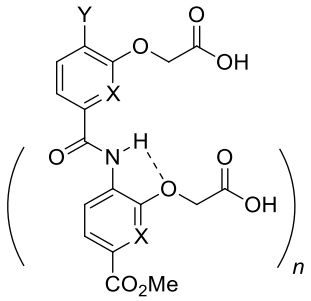
Table 2: Structures and IC_{50} values for compound library

Compound	R_1	R_2	R_3	IC_{50} [µM]
Helix 3 peptide		*Ac-GTEELLRALDQVNAAG-NH₂*		inactive[a]
31	*i*Bu	*i*Bu	*i*Pr	9.2 ± 0.9
32	*i*Pr	*i*Bu	*i*Bu	24 ± 1.6
33	Me	*i*Pr	*i*Bu	216 ± 16
34	*i*Bu	*i*Bu	*i*Bu	9.8 ± 1.3
35	*i*Bu	*i*Pr	*i*Bu	13 ± 1.5
36	benzyl	benzyl	benzyl	56 ± 6.0
37	*i*Pr	*i*Pr	*i*Pr	39 ± 4.0
38	*i*Bu	*i*Pr	*i*Pr	17 ± 0.7
39	benzyl	*i*Pr	*i*Pr	20 ± 0.8
40	2-hydroxyethyl	*i*Pr	*i*Pr	416 ± 64
41[b]	*i*Pr	*i*Pr	*i*Pr	inactive

(a) Up to > 250 µM, (b) N-alkylated scaffold

Amyloid protein surface:

Saraogi *et al*. (2010) proposed an alternative mechanism of amyloid inhibition in which small molecule helix mimetics interferes with the helix assembly process in islet amyloid polypeptide (IAPP) aggregation. They designed an oligopyridylamide scaffold based α- helix mimetics (**42a-e** and **43a-e**) (Fig. 14) containing four or five carboxy-terminated side chains and projects functionality on one face of the molecule in direct analogy to an α-helix. The **41a-e** used intramolecular hydrogen bonding to rigidify the backbone. Similarly, oligobenzamide series **42a-e** was synthesized to increase the greater conformational flexibility of the aryl-C(=O)bonds. The helix mimetics significantly reduced the rate of fibrillogenesis of IAPP under lipid-catalyzed conditions. Compound **41e** showed a dose-dependent inhibition and an IC_{50} of 8 µm was obtained. The molecules acted as agonists of amyloid formation in the absence of the lipid and compound **41e** showed two- to three-fold acceleration in the aggregation kinetics.



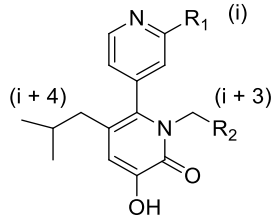
41a; X = N, Y = NO_2, n = 0
41b; X = N, Y = NO_2, n = 1
41c; X = N, Y = NO_2, n = 2
41d; X = N, Y = NO_2, n = 3
41e; X = N, Y = NHAc, n = 4

42a; X = C, Y = NO_2, n = 0
42b; X = C, Y = NO_2, n = 1
42c; X = C, Y = NO_2, n = 2
42d; X = C, Y = NO_2, n = 3
42e; X = C, Y = NHAc, n = 4

Figure 14: Oligopyridylamide scaffold-based α-helix mimetics (41 and 42)

Nuclear receptor box:

Becerril *et al*. (2007) designed and synthesized to mimic the surface functionality of an α-helical LXXLL motif also known as nuclear receptor box (where L is leucine and X is any amino acid including leucine) based on a pyridylpyridone scaffold. This short LXXLL sequence is necessary and sufficient for binding to the estrogen receptor (ER). Thus, ER expressed many essential ER-regulated genes. A series of substituted pyridylpyridone derivatives **(43-49)** was synthesized as shown in Fig. 15. The most compounds bound with K_i values in the low micromolar range in FP assay (Table 3). Compounds **45** and **48** containing one and two benzyl groups in the i and i+3 positions respectively, gave K_i values of 9.4 and 6.5 µm, respectively. Introduction of a more hydrophobic naphthyl group in **49** led to a further improvement with a K_i value of 4.2 µm.



43, R_1 = *i*Bu, R_2 = *i*Pr
44, R_1 = *i*Bu, R_2 = *i*Bu
45, R_1 = *i*Bu, R_2 = Bn
46, R_1 = *i*Bu, R_2 = $CH_2C(CH_3)_3$
47, R_1 = *t*Bu, R_2 = $CH_2C(CH_3)_3$
48, R_1 = Bn, R_2 = Bn
49, R_1 = Bn, R_2 = CH_2(2-naphthyl)

Figure 15: Pyridylpyridone scaffold-based α-helical mimetics and their FP assay results

Table 3: Results of the fluorescence polarization assay

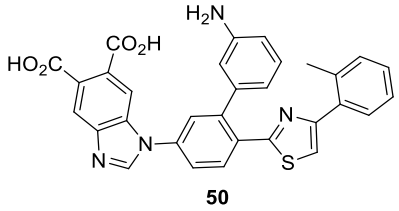
Compound	K_i(µM)	Compound	K_i(µM)
SRC-1 NR II	1.0 (0.3)	**46**	>50
43	34 (3)	**47**	>50
44	16 (3)	**48**	6.5 (0.5)
45	9.4 (2.0)	**49**	4.2 (0.5)

The values in brackets are the corresponding standard deviations.

Cdc42/Dbs protein surface:

Cummings *et al.* (2009) developed a more water-soluble 5-6-5 imidazole-phenyl-thiazole core-based novel α-helix mimetic **50** (Fig. 16), which mimic the Q770, K774, and L777 residues of Dbs, correspond to the *i*, *i* + 4, and *i* + 7 of a key Dbs α-helix. Cdc42 is a GTPase (guanine nucleotide triphosphatase) shown to mediate cancer cell resistance and also has been linked to diabetes, cardiovascular and neurodegenerative diseases (Sinha *et al*., 2008). Cdc42 is activated by interaction with the GEF (guanine nucleotide exchange factor), Dbs. A mant-GDP fluorescence-based activity assay showed the disruption of Cdc42/Dbs protein-protein

interaction at micromolar affinity (67 μM). A nonplanar conformation of diacid **50**, could match the positions of the *i, i* + 4, and *i* + 7 side chains in the key Dbs α-helix.



50

Figure 16: Imidazole-phenyl-thiazole core-based novel α-helix mimetic

CaM-smMLCK protein surface:

DeGrado *et al.* (2006) group synthesized arylamide scaffold-based synthetic inhibitors of CaM that are intended to mimic a CaM-binding helical peptide smMLCK (Crivivi *et al*., 1995) (Fig. 17**)**. CaM regulates the intracellular Ca^{2+} level to activation of a large number of regulatory proteins, including kinases, phosphatases, and ion channels and also plays important roles in many critical biological processes, such as inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, and short-term and long-term memory. Compound **51**, which has two D-Phe residues, showed a K_i value of 7.10 ± 1.48 nM in a fluorescence polarization assay, shown in Table 4. (^{1}H, ^{15}N)-HSQC NMR spectroscopy experiments proposed that compound **51** binds to CaM in a similar way as smMLCK does.

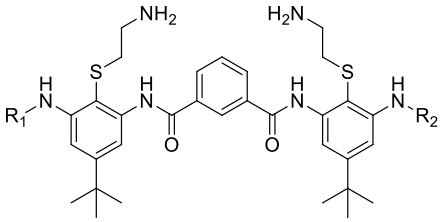


Figure 17: Arylamide scaffold-based helix mimetics inhibitors

Table 4: Results of the fluorescence polarization assay

Compounds	R_1	R_2	*K*i (nM)
51	D-Phe	D-Phe	7.10 ± 1.48
52	D-3-PyA	D-3-PyA	25.7 ± 3.42
53	D-2-Nal	D-2-Nal	83.4 ± 6.2
54	L-2-Nal	L-2-Nal	> 120
55	D-3-PyA	H	> 1200

PyA is pyridylalanine, Nal is naphthylalanine.

AKAP–PKA protein surface

Klussmann *et al*. (2012) developed polypyridines as helix mimetic for the inhibition of AKAP–PKA interactions (Fig. 18), taking into account the favorable solubility, and conformational orientation of the side chains residue, the hydrophobic and hydrophilic core of the binding cavity. Protein kinase A (PKA) phosphorylates a broad variety of substrates. Dysregulation of cellular processes that depend on AKAP–PKA interactions is associated with several diseases. The carboxy group of the external pyridine ring mimics the hydrophilic side chain of E300 and is likely to interact with Q4 of the D/D domain (Fig. 19). The mimetics **56b** and **56f** bound to the D/D domain with an estimated K_D=148 μM and K_D=31 μM, respectively

which obtained from isothermal titration calorimetry. The inhibitory potency of **56a** and **56f** was very low. SAR and NMR data suggested that **56b** bound with the D/D domain of RIIα subunits in which **56b** assumed a conformation that mimics the α-helical AKAP18d fragment.

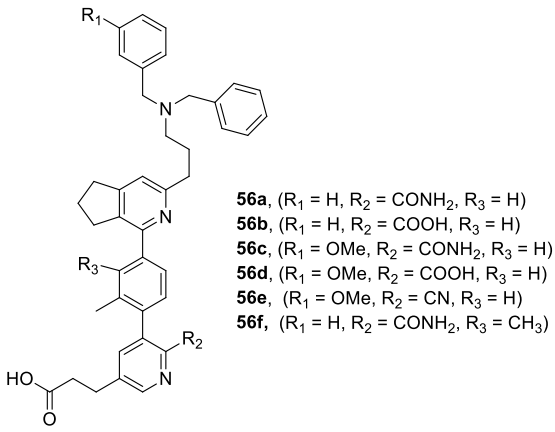


Figure 18: Polypyridines as helix mimetics inhibitors.

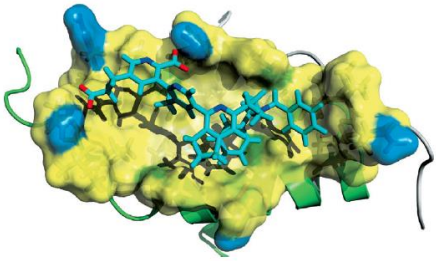


Figure 19: **Model of the interaction between terpyridine 56b (cyan) and the D/D domain of human regulatory RIIa subunits of PKA.** (**Klussmann *et al*., 2012**)

(KSHV Pr) dimerization surface:

Shahian *et al.* (2009) identified a small-molecule inhibitor of human Kaposi's sarcoma–associated herpesvirus (KSHV Pr) dimerization by screening a helical mimetic compound library (Fig. 20). Herpesvirusis one of the most prevalent viral families, including eight human types that cause a variety of devastating illnesses such as mononucleosis (Epstein-Barr virus, EBV), genital herpes (herpes simplex virus, HSV), shingles (varicella-zoster virus, VZV), retinitis (cytomegalovirus, CMV) and cancer (Kaposi's sarcoma–associated herpes-virus, KSHV). Inhibitor **1**(IC_{50} = 8.8 ± 0.3 μM) was identified after screening a library of 182 small-molecule helical mimetics. Further, seven structural analogs of **57** synthesized and screened all compounds using a fluorogenic activity assay. The most potent being **58**, which had an $IC_{50}$of 3.1 ± 0.2 μM, and **59**, IC_{50}= 16.4 ± 0.7 μM. The improved $IC_{50}$of **(58)**, may be due to the added pyridine nitrogen, which permits for either intermolecular hydrogen bonding to residues on the protease or intramolecular hydrogen bonding that rigidifies its structure. The inhibitor **58** inhibited protease dimerization by associating with interface residues, including Trp109, and Met197 and Ile201 assume the *i* and *i* + 4 positions on α-helix 5. Heteronuclear single quantum coherence (HSQC) spectra of selectively labeled ^{13}C-Met KSHV Pr revealed that increasing concentrations of **58** induced a chemical shift perturbation and an increase in the peak volume of the Met197-monomer resonance. At the same time, they observed a reduction in the peak volume of the Met197-dimer peak, which broadens beyond detection at 1 molar equivalence inhibitor (24 μM).

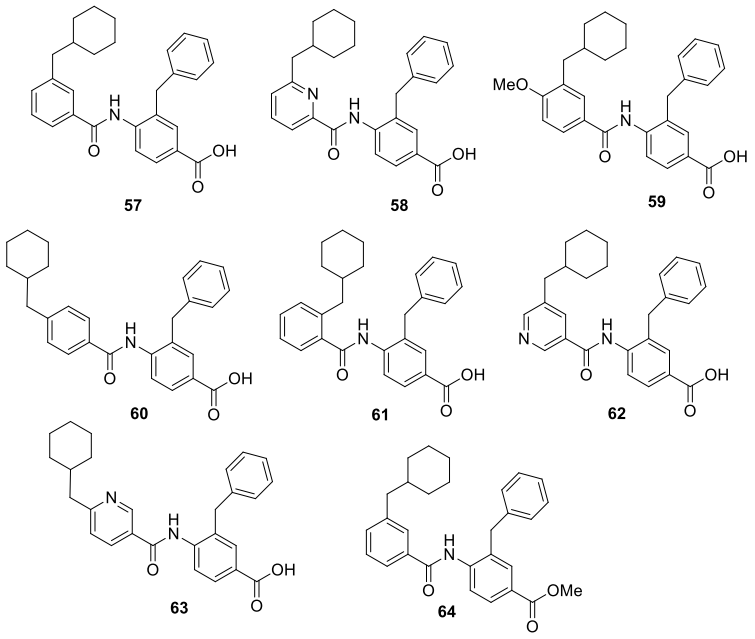


Figure 20: Compounds **58-64** are structural analogs of initial hit compound **57**.

Conclusions:

The examples of protein surface binders discussed here are based on small molecule α-helix mimetics. An important requirement of suitable molecular scaffolds is the specific orientation of the side chain residues as well as the critical balance of hydrophobic and charged residues. This will lead to complementarity and high affine interactions with a surface target, without resulting in insolubility or aggregation of these agents. Most of the examples discussed above on isolated proteins *in vitro* have been shown to interact strongly with the desired protein surfaces. Now, a key question is associated with the applications in medicinal-/pharmaceutical chemistry. To develop a clinical candidate, one must test these compounds under physiological conditions and ultimately *in vivo*. Therefore questions of pharmacokinetics, bioavailability, and toxicity have to be addressed. However, several examples included in this chapter show good activity in cell-based assays and few are even effective *in vivo*. There is still a need for further development to circumvent these challenges which will certainly continue to be a highly interesting field of investigation.

Abbreviations:

NK3- neurokinin 3; Phe-Phe Phenyl alanine-phenyl alanine; Bcl-xL-B-cell lymphoma-extra large; FP assay- fluorescence polarization assay; ITC- Isothermal *titration* calorimetry; PPI- protein–protein interaction; OHM- oxopiperazine helix mimic; HIF- hypoxia-inducible factor; IAPP- islet amyloid polypeptide; ER-Estrogen receptor; GEF- guanine nucleotide exchange factor; NMR-Nuclear magnetic resonance; HSQC- Heteronuclear single quantum coherence spectroscopy**;**

References:

1. Peczuh, M. W.; Hamilton, A. D., 2000. Peptide and Protein Recognition by Designed Molecules, *Chem. Rev*. 100 (7), 2479-2494.

2. Vallone, B.; Miele, A. E.; Vecchini, P.; Chiancone, E.; Brunori, M., 1998. Free energy of burying hydrophobic residues in the interface between protein subunits, *Proc. Natl. Acad. Sci. U.S.A.* 95(11), 6103-6107.
3. Clackson, T.; Wells, J. A., 1995. A hot spot of binding energy in a hormone-receptor interface, *Science.* 267(5196), 383-386.
4. Peterson, S. A.; Klabunde, T. K.; Lashuel, H. A.; Purkey, H.; Sacchettini, J. C.; Kelly, J. W., 1998. Inhibiting transthyretin conformational changes that lead to amyloid fibril formation, *Proc. Natl. Acad. Sci. U.S.A.* 95(22), 12956-12960.
5. Cochran, A. G., 2001. Protein–protein interfaces: mimics and inhibitors,*Curr. Opin. Chem. Biol.* 5(6), 654-659.
6. Azzarito, V.; Long, K.; Murphy, N. S.; Wilson, A. J., 2013. Inhibition of α-helix-mediated protein–protein interactions using designed molecules, *Nature Chem*, 5(3), 161-173.
7. Horwell, D. C.; Howson,W.; Ratcliffe, G. S.; Willems, H. M. G., 1996. The Design of Dipeptide Helical Mimetics: The Synthesis, Tachykinin Receptor Affinity and Conformational Analysis of 1,1,6-Trisubstituted Indanes, *Bioorg. Med. Chem.* 4(1), 33-42.
8. Kutzki,O.; Park, H. S.; Ernst, J. T.; Orner, B. P.; Yin, H.; Hamilton, A. D., 2002. Development of a Potent Bcl-xL Antagonist Based on r-Helix Mimicry, *J. Am. Chem. Soc.* 124(40), 11838-11839.
9. Yin, H., Lee, G.-i.; Sedey, K. A.; Rodriguez, J. M.; Wang, H.-G.; Sebti, S. M.; Hamilton, A. D., 2005. Terephthalamide Derivatives as Mimetics of Helical Peptides: Disruption of the Bcl-xL/Bak Interaction, *J. Am. Chem. Soc.* 127(15), 5463-5468.
10. Rodriguez,J. M.; Nevola,L.; Ross,N. T.; Lee, G.-i.; Hamilton, A. D., 2009. Synthetic Inhibitors of Extended Helix–Protein Interactions Based on a Biphenyl 4,4′-Dicarboxamide Scaffold, *ChemBioChem*, 10(5), 829 – 833.
11. Plante,J. P.; Burnley,T.; Malkova,B.; Webb,M. E.; Warriner,S. L.;Edwards, T. A.; Wilson,A. J., 2009. Oligobenzamide proteomimetic inhibitors of the p53–*h*DM2 protein–protein interaction, *Chem. Commun.*,34, 5091–5093.
12. Azzarito,V.; Miles, J. A.; Fisher,J.; Edwards,T. A.; Warriner, S. L.; Wilson, A. J., 2015. Stereocontrolled protein surface recognition using chiral oligoamide proteomimetic foldamers, *Chem. Sci*., *6*, 2434-2443.
13. Lee, J. H.; Zhang, Q.; Jo, S.; Chai, S. C.; Oh, M.; Im, W.; Lu, H.; Lim, H.-S., 2011. Novel Pyrrolopyrimidine-Based α-Helix Mimetics: Cell-Permeable Inhibitors of Protein-Protein Interactions, *J. Am. Chem. Soc.*, 133(4), 676–679.
14. Lao, B. B.; Grishaginb, I.; Mesallati, H.; Brewera,T. F.; Olenyukb, B. Z.; Arora, P. S., 2014. In vivo modulation of hypoxia-inducible signaling by topographical helix mimetics, *Proc Natl Acad Sci USA*, 111 (21),7531–7536.
15. Burslem,G. M.; Kyle, H. F.; Breeze, A. L.; Edwards,T. A.; Nelson,A.; Warriner, S. L.; Wilson, A. J., 2014. Small-Molecule Proteomimetic Inhibitors of the HIF-1α–p300 Protein–Protein Interaction, *ChemBioChem*, 15(8), 1083 – 1087.

16. Saraogi, I.; Hebda, J. A.; Becerril, J.; Estroff, L. A.; Miranker, A. D.; Hamilton Andrew,A. D. 2010. Synthetic α-Helix Mimetics as Agonists and Antagonists of Islet Amyloid Polypeptide Aggregation. *Angew. Chem. Int. Ed*, 122(4), 748-751.
17. Becerril, J.; Hamilton,A. D., 2007. Helix Mimetics as Inhibitors of the Interaction of the Estrogen Receptor with Coactivator Peptides, *Angew. Chem. Int. Ed*, 119(24), 4555 – 4557.
18. Cummings, C. G.; Ross, N. T.; Katt, W. P.; Hamilton, A. D., 2009. Synthesis and Biological Evaluation of a 5-6-5 Imidazole-Phenyl-Thiazole Based α-Helix Mimetic, *Org. Lett., 11(1)*, 25-28.
19. Sinha, S.; Yang, W., 2008. Cellular signaling for activation of Rho GTPase Cdc42, *Cell. Signalling*, 20(11), 1927–1934.
20. Yin, H.; Frederick, K. K.; Liu,D.; Wand,A. J.; DeGrado, W. F., 2006. Arylamide Derivatives as Peptidomimetic Inhibitors of Calmodulin, *Org. Lett.*, 8(2), 223-225.
21. Crivici, A.; Ikura, M., 1995. Molecular and Structural Basis of Target Recognition By Calmodulin, *Annu. Rev. Biophys. Biomol.Struct*. *24*, 85-116.
22. Tröger, J.; Moutty, M. C.; Skroblin, P.; Klussmann, E., 2012. A-kinase anchoring proteins as potential drug targets. *Br. J. Pharmacol.* 166(2), 420–433.
23. Shahian,T.; Lee,G. M.; Lazic,A.; Arnold,L. A.; Velusamy,P.; Roels,C. M.; Guy,R. K.; Craik,C. S., 2009. Inhibition of a Viral Enzyme by a Small Molecule Dimer Disruptor, *Nat. Chem. Biol*, 5(9), 640-646.

RECENT TRENDS OF CELL PENETRATING PEPTIDES IN BIOMEDICAL AND PHARMACEUTICAL RESEARCH- A CONCISE REVIEW

Moumita Saha[1] and Abhijit De*[2]

[1] Department of Pharmaceutical Quality Assurance,
Manipal College of Pharmaceutical Sciences,
Manipal University, Manipal, Karnataka-576104

[2] Department of Pharmacology,
Bengal School of Technology, Sugandha, Hooghly, West Bengal-712102

*Corresponding author E-mail: abhijit@bstpharmacy.in

Abstract:

Cell penetrating peptides (CPPs) are small amino acid sequences that can enhance the transportation and cellular uptake of various bio-active cargoes that are unable to cross the cell membrane, which was a major obstacle to efficient intracellular delivery for present therapeutics. It acts as a carrier for various cargoes like nucleic acid, proteins, siRNA, and therapeutic agents both *in-vivo* and *in-vitro*. The present therapeutics are difficult to reach their cellular or intracellular target sites for treatment, which is a great challenge. To overcome the challenge, a new concept of Cell penetrating peptides (CPPs) is discovered after extensive research. Hundreds of sequences of different CPPs have been classified based on their physico-chemical properties and origins, e.g. penetratin, transportan and HIV-Tat are very common variants. The endocytotic pathway and membrane translocation via direct penetration are the most suggested uptake mechanisms to gain better cell penetration efficiency. The CPPs have a wide range of advantages, among which are rapid and potent delivery, good efficiency, and low toxicity in comparison with other carriers used so far. But there are low specificity and instability issues, though these can be eliminated by various approaches like nanocarriers, liposomes, inorganic agents, polymeric particles, etc. To overcome the low specificity, the CPP-cargo complexes may be conjugated with another moiety, which brings cell or tissue specificity. They can be applied to many diseases like cancer therapy, tumour therapy, rheumatoid arthritis, asthma, ischemia, etc. They can also be used in different fields like cell imaging, gene editing, and diagnosis. The researchers are trying their best to employ them in other fields of medical science. In this review paper, we are focusing on the recent advancements in the potential usage of CPPs.

Keywords: Cargoes; Gene editing; Intracellular delivery; Liposomes; Nano-carriers

Introduction:

The plasma membrane protects eukaryotic cells from unregulated influxes of biologically active molecules, except small hydrophobic molecules (Stewart *et al.,* 2008). Strongly polar, poorly soluble hydrophilic compounds are transported by transporters to the cytoplasm via an energy-dependent mechanism (Reissmann *et al.,* 2014). Cell penetrating compounds like cell penetrating peptides (CPPs) can penetrate into the cell via energy-dependent as well as energy-independent processes to deliver different cargoes into the cell (Böhmová *et al.,* 2018).

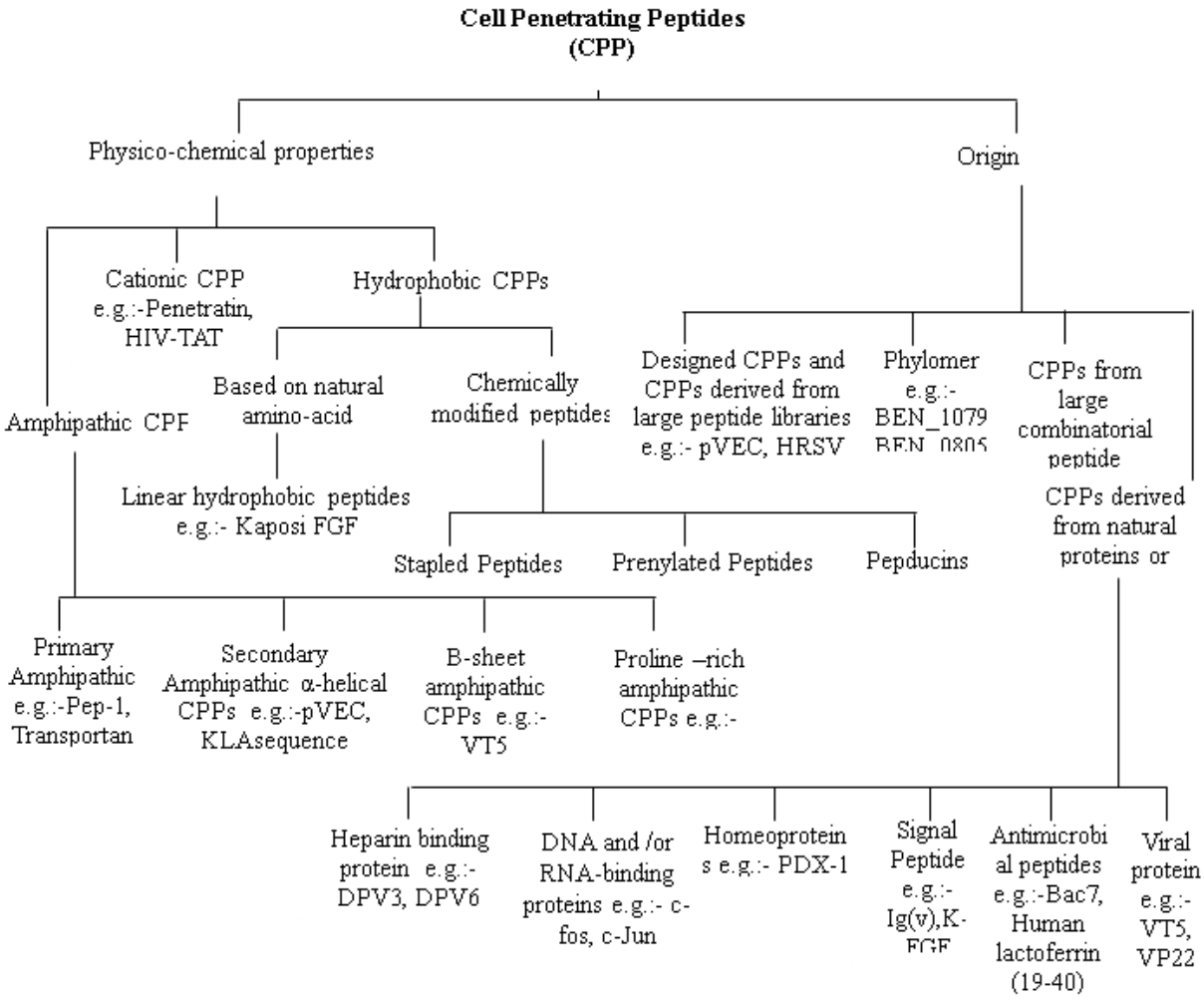
CPPs are usually oligopeptides consisting of 5–30 amino acids that can penetrate the cell membrane without any energy-dependent process (Reissmann *et al.,* 2014). CPPs are also called

membrane translocation sequences, protein translocation domains, or Trojan horse peptides. Generally, CPPs are short, water-soluble, partly hydrophobic polybasic peptides with a net positive charge in physiological pH-1 (Madani *et al.*, 2011).

The main advantages of CPPs are good efficiency due to rapid and potent delivery, low cytotoxicity in comparison with most drug carriers, and their ability to penetrate into the cytoplasm at low micromolecular concentrations both in vitro and in vivo without any chiral receptor or significant membrane damage. However, they lack cell-type selectivity, the impossibility of oral administration, and a low plasma half-life as a result of enzyme degradation and renal filtration (Böhmová *et al.*, 2018). Higher cell-type selectivity can be obtained using cell-specific or non-specific CPP with a transport cargo or by conjugating it to another moiety for targeted activity (Milletti *et al.*, 2012).

CPPs have been developed for applications in cell imaging and gene editing. It has been used as a vector of gene expression like oligonucleotides for antisense, decoy dsDNA (double-stranded DNA) applications, siRNA (small interfering RNA), and as a transfection agent for plasmid delivery. The therapeutic application of CPP includes the transportation of compounds through the skin, blood-brain barrier (BBB), conjunctiva of the eyes, and other affected tissues or organs. It can also detect tumours and their metastases (Reissmann *et al.*, 2014).

Classification of CPPs



CPPs, derived from various natural proteins or chemical sequences, can be classified on the basis of their physico-chemical properties or origin. In physico-chemical property-based classification, cationic CPPs derived from HIV-1 protein TAT are made of multiple lysine and arginine residues (Böhmová *et al.,* 2018); amphipathic CPPs contain both cationic and anionic peptides. It can be further classified based on the type of amino acid present (Deshayes *et al*., 2006). Another class is hydrophobic CPP, which contains a polar residue having a low net charge or a hydrophobic motif or chemical group that is essential for uptake (Milletti *et al.,* 2012). In origin-based classification, mainly structural-activity studies of CPP are concerned. The chart below gives a general idea on classification of CPPs.

Uptake process of CPP

The uptake mechanism of CPP includes interaction with the membrane surface or with the phospholipid bilayer directly or disturbing local membrane and intracellular processes leading to further changes in the membrane structure to internalise into endosomes or the cytosol. Most of the CPPs uptake mechanisms involve (i) direct penetration or energy-independent pathways; (ii) energy-dependent pathways or endocytotic pathways. There are some CPPs that still have an undiscovered uptake mechanism (Reissmann *et al.,* 2014).

Direct Penetration or Energy-Independent Mechanism:

This process involves the interaction of positively charged CPP with negatively charged cell membrane components, e.g., the interaction between heparan sulphate and the phospholipid bilayer. Direct penetration can take place even at low temperatures (4 oC). Various mechanisms are involved in the direct penetration of CPP into the cytosol (Böhmová *et al.,* 2018). They are-

Involvement of proteoglycans: ECM contains proyeoglycans having a core protein with which one or more glycosaminoglycan (GAG) chains are covalently bound (Couchman *et al*., 2003). CPP binding results in clustering of GAGs, which triggers intracellular signalling, cytoskeleton remodelling, and finally cellular uptake.

Barrel stave model: It involves the helix formation of CPP. In the helix structure, the hydrophobic residue faeces towards the hydrophobic tails of the lipid bilayer, and the hydrophilic residue forms the internal environment of the pore (Brogden *et al*., 2005; Böhmová *et al.,* 2018).

Toroidal pore model: It also depends on the helical structure and pore formation, though the mechanism is different. The pores are formed with peptides in association with the polar heads of the lipids inside the cell membrane. The hydrophilic core of the pore is lined with inserted peptides and the hydrophilic head of the cell membrane. Magainins and protegrins are peptides that show toroidal pores (Matsuzaki *et al.,* 1996; Böhmová *et al.,* 2018).

Carpet-like model: This model contains peptides parallel to the membrane surface. In this model, interaction occurs between an anionic phospholipid head group and positively charged peptides. The CPP concentration must be higher than the threshold concentration to form a carpet-like coating. After a sufficient amount of membrane coating, membrane permeation occurs. Binding on the hydrophilic group of the peptide with the phospholipid head group results in rotation, which leads to destabilisation and reorganisation of the cell membrane. The lipid bilayer transforms into a micelle, which forms a transient hole. (Khandia *et al.,* 2017; Böhmová *et al.,* 2018)

Inverted micelle model: It is formed in a hexagonal structure between two cell membrane bilayers, in which CPP is surrounded by a hydrophobic part of the membrane. Interaction between the positively charged CPP and negatively charged cell membrane, as well as interaction between the hydrophobic part of the CPP and the hydrophobic part of the membrane, takes place (Guo *et al.,* 2016; Islam *et al.,* 2018). The HIV-1 Tat peptide can be internalised by this model formation. (Khandia *et al.,* 2017; Böhmová *et al.,* 2018)

Endocytosis or energy-dependent mechanism:

It includes phagocytosis and pinocytosis processes for the uptake of CPP. Phagocytosis is a complex process for the engulfment of large particles like macrophages and neutrophils (Stewart *et al*., 2008).

Pinocytosis occurs in all types of cells. It can be further divided into four pathways. They are-

Macropinocytosis: They form vesicles during the inner folding of the plasma membrane called macropinosomes. This pathway is used by a variety of CPP-cargo complexes (Böhmová *et al.,* 2018).

Clathrin-mediated endocytosis: The invagination of the membrane and formation of vesicles coated with clathrin protein take place, e.g. Tat is conjugated with fluorophore (Böhmová *et al.,* 2018).

Caveloae/lipid raft-mediated endocytosis: Membrane invagination and small vesicles coated with caveolae proteins occur in this pathway, e.g., Tat is conjugated with protein (Böhmová *et al.,* 2018).

Clathrin and caveolae-independent endocytosis (Stewart *et al*., 2008)

The extracellular molecules are encapsulated into lipid vesicles and internalised into the cell. The limitation of this process is that the solute molecules have to escape the vesicle before they are transported back to the plasma membrane or fused with lysosomes. The CPP-cargo complex reacts with the phospholipid of the endosomal membrane, causing the release of CPP (Stewart *et al*., 2008). Multivalent CPPs endosomolytic activity through strong interaction with the cell membrane is greater than that of monomeric CPPs and they escape more efficiently. Cyclization of argentine-rich CPPs also has more effective cellular uptake (Böhmová *et al.,* 2018).

Targeted sub-cellular localization: -

The process involves direct transportation of CPP into the cell organelles by recognition of specific localization sequences, like targeting nucleic acid for gene therapy in cancer or targeting mitochondria (Böhmová *et al.,* 2018).

Cell and tissue selectivity:

The lack of cell-specificity of first-generation CPP can be nullified by internalisation helpers like hormone analogues, e.g., ligands of GPCRs and ligands of integrin. Further tissue selectivity was achieved by the insertion of homing sequences in the fusion protein of the CPP-cargo complex. Further development occurred in a protease-active-able CPP that acts on membrane-bound secreted proteases of tumour cells and targets specifically tumour cells. The formation of pH-sensitive CPPs can be another approach (Reissmann *et al.,* 2014).

CPPs and AMPs:

Antimicrobial peptides (AMPs) are involved in host innate immunity. They have structural similarities with CPP; when it binds to a target membrane, it forms an amphipatic structure. The cationic residue present in it enhances the electrostatic binding to the target cell membrane, and the hydrophobic residue drives the entry through the lipid bilayer. (Madani *et al.*, 2011)

Factors affecting the Mechanism of cellular uptake: -

In cellular uptake mechanism study**,** both physico-chemical study of CPP and utilized experimental conditions are important

i. **Structure**
ii. **Concentration**
iii. **Thermodynamic binding**
iv. **Hydrophobicity**
v. **Cargo**
vi. **Cysteine and disulphide bridges**
vii. **Fusogenic extension**
viii. **Influence of detection syetem**

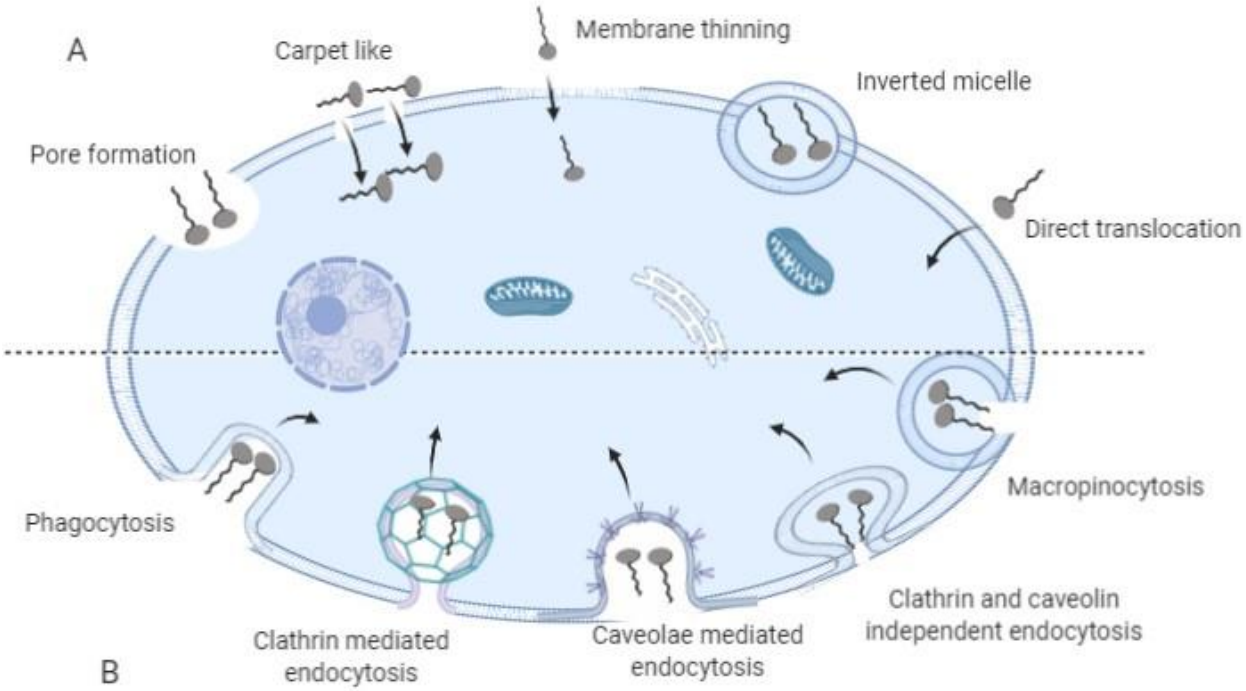


Figure 1: Mechanism of CPP entry into target cell. (A) Energy independent pathway (B) Energy dependent pathway

Delivery of CPP into cells

There are some approaches to increasing the permeability of the cargo inside the cell. A large number of cargoes can be efficiently transported both *in vivo* and *in vitro*. Depending on their properties, they can be classified into three major classes (Koren *et al.*, 2012). They are-

1. **Nano-carriers:-**

There are many types of nanocarriers. They are-

1. **Lipid-based nano-carriers:** They are artificial phospholipid vesicles that can be loaded with a variety of agents. 200-nm liposomes can be delivered intracellularly with a Tat peptide attached to their surface (Torchilin *et al.*, 2001).
2. **Polymeric nano-carriers: CPP-m**modified micelles can be used to enhance the intracellular activity of large, poorly soluble biologically active agents. It can be designed by combining pDNA complexes with TAT-modified chitosan that have effective transfection efficiency (Rahamat *et al.*, 2012).
3. **Inorganic nano-carriers:** A 100-times higher efficiency was found by intra-cellular magnetic labelling of lymphocyte cells using dextran-coated super-paramagnetic iron oxide nano-particles (SPIONs) (Koren *et al.*, 2012).
4. **Stimulus-responsive smart nano-carriers:** Due to non-specificity and toxicity in the first phases of nanocarrier development, a new approach was taken by sterically shielding a targeting antibody or a polymer. After accumulation on the target, the protective moiety will detach and reveal the CPP on the target (Koren *et al.*, 2012).
5. **Dendrimers:** Dendrimers are macromolecules with a focal core and a peripheral layer of different functional groups. Linear CPPs can be delivered through dendrimers by designing into multi-branched moiety. The advantages of dendrimers are higher cell penetration, low toxicity and hemolysis, and higher serum stability over linear CPPs (Reissmann *et al.*, 2014). Poly-amidoamine (PAMAM) dendrimers and TAT peptide were attached with bacterial magnetic nanoparticles (BMPs) to form a trans-membrane targeted siRNA delivery system in gene therapy of brain tumours.
6. **Micelles:** CPP can also be delivered via polymeric micelles in the brain with TATp as a promoter, which can be achieved via intranasal administration (Kanazawa *et al.*, 2011). Also, a PEG-modified phospholipid micelle coating strategy is designed to protect SPIONs for magnetic resonance imaging (MRI) (Nitin *et al.*, 2004).
7. **Cargoes:** It consists of a low-molecular-weight drug or macromolecular delivery system, including targeting ligands and diagnostic lebels.
8. **Drugs, peptides, and proteins: cargoes** are used for transportation of cisplatin and other organometallic compounds, anticancer peptide vaccines, or other anticancer drugs or chaperons into the tumour cells. Anti-Alzheimer drugs can also be transported into target cells via CPPs (Arbel *et al.*, 2007). Bcl-xL-protein protects the heart and brain from ischemic injuries (Cao *et al.*, 2002).
9. **Nucleotides and oligo-nucleotides:** Labelled nucleoside phosphates like GTP analogues help in studying GTP-labelled intracellular reactions (Mussbach *et al.*, 2011). Peptide nucleic acids (PNAs) or splice-correcting oligo-nucleotides are also internalised for research and clinical study purposes (Reissmann *et al.*, 2014).
10. **DNA and plasmids:** Plasmid DNA and linear DNA can transfect into cells and express coded proteins (Schlenk *et al.*, 2013).

11. **Imaging agents: imaging** agents are mainly used to visualise physiological structure and internal features of living organisms and observe in *vivo* cellular function (Stewart *et al.,* 2008). Fluorophores, quantam dots, gold nanoparticles, etc. are used as imaging agents.

Biomedical application

Therapeutic agents like proteins, drug carriers, and peptides are required to reach inside the cell via active transport mechanisms. CPPs can carry different cargoes inside the cell without causing cellular damage. The main advantages of CPPs are that they have low cytotoxicity and no limitation on cargo types to attach (Heitz *et al.,* 2009). Thus, there are wide varieties of biomedical applications of CPP (Derakhshankhah *et al.,* 2018). They are

1. **Anti-microbial and anti-fungal action:** CPPs can affect the membranes of bacteria and fungi. They are similar in size to anti-microbial peptides and also similar in positive charges, which increase interactions with biomembranes having negative charges. Multiple guanidinum groups of CPPs enhance the uptake as well as anti-microbial activity. The CPP-PNA conjugate has an efficient antisense effect on essential mRNA and rRNA sequences, which prohibit gene expression as well as the growth of bacterial cells encapsulated in macrophages (Ma *et al.,* 2014). Also, CPPs like Tat and Penetratin have anti-fungal activities (Derakhshankhah *et al.,* 2018). Two analogues of penetratin have shown inhibitory effects against *Cryptococcus neoformans*. A human antimicrobial CPP, Histatin 5, found in human saliva can be used for selective delivery of cargo into the mitochondria of fungi and protozoa (Kardani *et al*., 2019).
2. **Anti-inflammatory action:** Nuclear factor-kB, a protein complex, regulates the transcription of inflammation-related genes like chemokines, cytokines, and inflammatory enzymes (Kanemaru *et al.,* 2011). Abnormal activation of these proteins causes viral infections and inflammatory bowel diseases. A CPP named AIP6 interacts with this protein and shows anti-inflammatory activity.
3. **Tumour therapy:** CPP, along with therapeutic agents, can increase uptake and treatment efficiency in tumour cells. Several strategies were taken in target moiety to improve the treatment efficiency of CPPs, like cell-targeting peptides, active-able cell penetrating peptides, and transducible agents in chemotherapic treatment that attached with target moieties (Kanemaru *et al.,* 2011).
4. Cell-targeting peptides are combinations of CPPs and tumour-targeting peptides.
5. Active-able CPPs are polycationic CPPs connected with cleavable linkers with a neutralising polyanion (Kanemaru *et al.,* 2011). These CPPs also help in imaging intracellular enzymatic reactions, which helps in targeting enzyme-associated cancer (Lee *et al.,* 2014).
6. Various p53-derived peptides covalently attached to Tat or penetratin can restore tumour suppressor functions in cancer cells.

TATp-lipoplexes enhance the delivery of the plasmid pEGFP-N1 into U-87 MG tumour cells *in vitro* (Kale *et al.,* 2007). Tatp-modified micelles have shown increased cytotoxicity in cancer cells both *in vitro* and *in vivo* (Sawant *et al.,* 2009). Poly-amidoamine (PAMAM) dendrimers and TAT peptide were attached with bacterial magnetic nanoparticles (BMPs) to form a trans-membrane targeted siRNA delivery system in gene therapy of brain tumours.

1. **Vaccine:** The combination of natural adjuvant (Hsp27) with CPPs (HR9 and Cady-2) could considerably stimulate effective immune responses, which is a promising approach in the development of HCV therapeutic vaccines (Alizadeh *et al*., 2019). The use of M918 and MPG CPPs as proteins as well as gene carriers develops HIV-1 Nef-specific B- and T-cell immune responses, which is a promising approach to developing an HIV-1 monovalent vaccine (Rostami *et al*., 2019).
2. **Transplant rejection:** A key factor in transplant rejection is the proto-oncogene c-Myc, expressed in different tissues, including the liver, lung, and cornea. Recently, CPPs have been used for the treatment of transplant rejection. Hosseini showed the ability of a novel compound named AVI-5126 peptide (formulation: c-Myc antisense PMO linked to an arginine-rich CPP) to prevent corneal rejection in rats (Hosseini *et al.,* 2012).
3. **Ocular medication:** Recently, CPP for ocular delivery was designed to transport small molecules like fluorescent dyes into retinal cells both in vitro and in vivo. Studies showed that HIV Tat and HSV VP22 CPPs can deliver recombinant proteins to human embryonic retinoblasts only in vitro (Bolhassani *et al.,* 2017).
4. CPPs are also used for the delivery of siRNA into cultured cells via direct complexation with the siRNA anionic phosphate backbone. Linking siRNA with transportan or penetratin has shown a slicing response. Slicing of the vascular endothelial growth factor gene (VEGF) can effectively treat cancer. It has been observed that MPG peptide can improve the delivery of siRNA into cells (Farkhani *et al.,* 2014).
5. CPP complexes with therapeutic cargoes can be used in mascular dystrophy. Antigen oligo-nucleotides, which cause exon skipping by a specific mRNA sequence binding, are used in Duchenne muscular dystrophy (Durzynska *et al.,* 2015).
6. CPP conjugated with anti-appototic peptides shows therapeutic action, just as BH4 peptide inhibitors of the mitochondrial apoptotic pathway show cardio-protective properties in cases of heart infraction (Boisguerin *et al.,* 2013). Internalisation of peptide inhibitors of c-Jun N-terminal kinase (Borsello *et al.,* 2003) or anti-apoptotic peptides like BH4 (Boisguerin *et al.,* 2011) and Bcl-xL-protein (Cao *et al.,* 2002) protects the heart and brain from ischemic injuries.
7. CPP-cargo complexes are used to increase apoptosis in drug-resistant cells or improve the cytostatic agent's effect. CPPs can also be used as transporters of contrast agents for labelling tumour cells in the diagnosis of cancer (Copolovici *et al.,* 2014).
8. A CPP-mediated immune response was studied to overcome bacterial sepsis. CPPs were used to transport antiapoptotic proteins for the treatment of sepsis. It was found that in vivo injection of Bcl-xL or its BH4 domain conjugated with Tat reduced sepsis (Fonseca *et al*., 2009).
9. Small chemotherapeutic drugs (doxorubicin, methotrexate, and paclitaxel) can be delivered by CPP (Stewart *et al.,* 2008). It also helps to improve chemotherapeutic methods for preventing drug resistance in cells. Methotrexate, along with YTA2 CPP, shows five times more cytotoxicity in breast cancer (Lindgren *et al.,* 2006).
10. Tat peptides are attached to modular antigens for delivery and are used for the treatment of allergies and vaccine production (Kinyanju*i et al.,* 2008; Jiang *et al.,* 2014).

11. Tat or Pep-1 fused with superoxide dismutase protects pancreatic cells against oxidative stress (He *et al.*, 2014).
12. Administration of CPP penetratin along with insulin increases the bioavailability of intertinal and nasal insulin to 35% and 50%, respectively (Kamei *et al.*, 2013). It was seen that modifying the surface of nanoparticles through CPPs is a potential method for improving the absorption and delivery of insulin.
13. Other than these, it can also be used in diseases like rheumatoid arthritis, asthma, and ischemia (Derakhshankhah *et al.*, 2018).

Clinical status

Several preclinical studies are running to search for an effective model for different therapeutic uses like cerebral ischema, myocardial injury, cancer, ALS, cardiology, anti-prion treatment, and both bacterial and viral infections (Copolovici *et al.*, 2014).

1. RI-TAT-p53c protein was developed to restore the pro-apoptic activity of p53 protein, which has effects on cell cycle arrest and oncogenic stress (Vousden *et al.*, 2002; Snyder *et al.*, 2004).
2. CPP-DNA complexes are more stable in extra-cellular space in comparison to naked DNA, which, like pulmonary treatment, plasmid DNA along with TAT-PEG-polyethylene imine exhibits. It has about 600% higher transfection efficiency *in vivo* compared with plasmid DNA (Böhmová *et al.*, 2018).
3. CPP attached to siRNA has improved delivery efficiency and stability *in vivo*. MPG-8 targets cyclin B1 and prevents tumour growth in animals (Böhmová *et al.*, 2018).

More than 25 clinical trials on CPPs are in progress, including some agents in phase III (Lecher *et al.*, 2017).

The following CPP-related resources are currently available in online mode:

- **CellPPD: -** It is a prediction server that runs on Gautam's support vector machine. Penetration can be accessed via the implemented tools capability to detect mutants of one given peptide with a single mutation, scan a protein used for putative CPPs and CPP-related motifs, and analyse a fasta-formatted sequence batch. Of note, the authors also decided on full access to the data sets used in the training procedure. Prediction services and raw data are both available at http://www.imtech.res.in/raghava/cellppd (Gautam *et al*., 2013).
- **CPPpred:** This server is for predicting automatic CPP on the basis of Holton's N-to-1 artificial neural networking method, which is available at http://bioware.ucd.ie/testing/biowareweb/Server Pages/cpppred.php. (Holton *et al*., 2013).
- **CPPsite: -** It is a comprehensively annotated database of CPPs with a built-in analysis set. The database stores information on structure, sequence, basic physicochemical properties, literature or patent cross-references, and cell lines used for testing. Implemented tools allow exploration of CPP sequences in various sequences of proteins and calculation of their basic physical properties, which are available at http://crdd.osdd.net/raghava/cppsite (Gautam *et al*., 2012).

Conclusion:

In this review, various aspects of CPPs, like their physicochemical properties, cellular uptake mechanism, membrane translocation, and biomedical applications, are summarised.

After the discovery of CPPs, researchers have been attracted to the cell-penetrating properties of these compounds in various fields. Their wide spectrum, enhanced penetration, and mechanism of action are applied in various diagnostic and drug delivery applications. Recent advancements in the study of solid-phase peptide synthesis and the availability and affordability of various synthetic peptides permit the generation of more designed CPP sequences. The intracellular delivery of these CPPs can help achieve better drug bioavailability and therapeutic efficiency.

There are some limitations to CPP, such as low cell specificity, toxicity, inactivation by proteases, etc. Despite these limitations, CPP has a better future in the fields of treatment, diagnosis, etc.

References:

1. Alizadeh, S., Irani, S., Bolhassani, A. 2019. Simultaneous Use of Natural Adjuvants and Cell Penetrating Peptides Improves HCV NS3 Antigen-Specific Immune Responses. *Immunol Lett*, 212:70–80.
2. Arbel, M., Solomon, B. 2007. Immunotherapy for Alzheimer's disease: Attacking Amyloid-B From the Inside. *Trends Immunol*, 28:511–513.
3. Böhmová, E., Machová, D., Pechar, M., Pola, R., Venclíková, K., Janoušková, O., Etrych, T. 2018. Cell-Penetrating Peptides: A Useful Tool for the Delivery of Various Cargoes into Cells. *Physiol Res*, 65(2):S267-S279.
4. Boisguerin, P., Giorgi, JM., Barrère-Lemaire, S. 2013. CPP-Conjugated Anti-Apoptotic Peptides as Therapeutic Tools of Ischemia-Reperfusion Injuries. *Curr Pharm Des*, 19:2970–2978.
5. Boisguerin, P., Red-Clouet, C., Franck-Miclo, A., Licheheb, S., Nargeot, J., Barrere-Lemaire, S., Lebleu, B. 2011. Systemic Delivery of BH4 Anti-Apoptotic Peptide Using Cpps Prevents Cardiac Ischemia-Reperfusion Injuries In Vivo. *J Control Rel*, 156:146–153.
6. Bolhassani, A., Jafarzade, BS., Mardani, G. 2017. In Vitro and In Vivo Delivery of Therapeutic Proteins Using Cell Penetrating Peptides. *Peptides*, 87:50–63.
7. Borsello, T., Clarke, PGH., Lorenz, H., Vercelli, A., Repici, M., Schoderet, DF., Bogousslavsky, J., Bonny, C. 2003. A Peptide Inhibitor of C-Jun N-Terminal Kinase Protects Against Excitotoxicity and Cerebral Ischemia. *Nat Med*, 9:1180–1186.
8. Brogden, KA. 2005. Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria?. *Nat Rev Microbiol*, 3:238-250.
9. Cao, G., Pei, W., Ge, H., Liang, Q., Luo, Y., Sharp, FR., Lu, A., Ran, R., Graham, SH., Chen, J. 2002. In Vivo Delivery of Bcl-Xl Fusion Protein Containing the TAT Protein Transduction Domain Protects Against Ischemic Brain Injury and Neuronal Apoptosis. *J Neurosci*, 22:5423–5431.
10. Copolovici, DM., Langel, K., Eriste, E., Langel, Ü. 2014. Cell-Penetrating Peptides: Design, Synthesis, and Applications. *ACS Nano*, 8:1972–1994.

11. Couchman, J.R. 2003. Syndecans: Proteoglycan Regulators of Cell-Surface Microdomains?. *Nat Rev Mol Cell Biol*, 4:926-937.
12. Derakhshankhan, H., Jafari, S. 2018. Cell Penetrating Peptides: A Concise Review with Emphasis on Biomedical Applications. *Biomedicine & Pharmacotherapy*, 108:1090-1096.
13. Deshayes, S., Morris, MC., Divita, G., Heitz, F. 2006. Interactions of Amphipathic Cpps with Model Membranes. *Biochim Biophys Acta*, 1758:328–335.
14. Durzyńska, J., Przysiecka, Ł., Nawrot, R., Barylski, J., Nowicki, G., Warowicka, A., Musidlak, O., Goździcka-Józefiak, A. 2015. Viral and Other Cell-Penetrating Peptides as Vectors of Therapeutic Agents in Medicine. *J Pharmacol Exp Ther*, 354**:**32-42.
15. Farkhani, SM., Valizadeh, A., Karami, H., Mohammadi, S., Sohrabi, N., Badrzadeh, F. 2014. Cell Penetrating Peptides: Efficient Vectors for Delivery of Nanoparticles, Nanocarriers, Therapeutic and Diagnostic Molecules. *Peptides*, 57:78–94.
16. Fonseca, SB., Pereira, MP., Kelley, SO. 2009. Recent Advances in the Use of Cell-Penetrating Peptides for Medical and Biological Applications. *Adv Drug Deliv Rev*, 61:953–964.
17. Gautam, A., Chaudhary, K., Kumar, R., Sharma, A., Kapoor, P., Tyagi, A., Raghava, GP. 2013. Open Source Drug Discovery Consortium in Silico Approaches for Designing Highly Effective Cell Penetrating Peptides. *J Transl Med*, 11:74.
18. Gautam, A., Singh, H., Tyagi, A., Chaudhary, K., Kumar, R., Kapoor, P., Raghava, GP. 2012. Cppsite: A Curated Database of Cell Penetrating Peptides. *Database (Oxford)*, 2012:bas015.
19. Guo, Z., Peng, H., Kang, J., Sun, D. 2016. Cell-Penetrating Peptides: Possible Transduction Mechanisms and Therapeutic Applications. *Biomed Rep*, 4:528-534.
20. He, XH., Yan, XT., Wang, YL., Wang, CY., Zhang, ZZ., Zhan, J. 2014. Transduced PEP-1-Heme Oxygenase-1 Fusion Protein Protects against Intestinal Ischemia/Reperfusion Injury. *J Surg Res*, 187:77–84.
21. Heitz, F., Morris, MC., Divita, G. 2009. Twenty Years of Cell-Penetrating Peptides: From Molecular Mechanisms to Therapeutics. *Br J Pharmacol*, 157:195–206.
22. Holton, TA., Pollastri, G., Shields, DC., Mooney, C. 2013. Cpppred: Prediction of Cell Penetrating Peptides. *Bioinformatics*, 29:3094–3096.
23. Hosseini, A., Lattanzio, FA., Samudre, SS. 2012. Efficacy of a Phosphorodiamidate Morpholino Oligomer Antisense Compound in the Inhibition of Corneal Transplant Rejection in a Rat Cornea Transplant Model. *J Ocul Pharmacol Ther*, 28:194–201.
24. Islam, MZ., Sharmin, S., Moniruzzaman, M., Yamazaki, M. 2018. Elementary Processes for the Entry of Cellpenetrating Peptides into Lipid Bilayer Vesicles and Bacterial Cells. *Appl Microbiol Biotechnol*, 102:3879-3892.
25. Jiang, Y., Li, M., Zhang, Z., Gong, T., Sun, X. 2014. Cell-Penetrating Peptides as Delivery Enhancers for Vaccine. *Curr Pharm Biotechnol*, 15:256–266.
26. Kale, AA., Torchilin, VP. 2007. Enhanced Transfection of Tumor Cells In Vivo Using 'Smart' Ph-Sensitive TAT-Modified Pegylated Liposomes. *J Drug Target*, 15:538–545.
27. Kamei, N., Nielsen, EJ., Khafagy, S., Takeda-Morishita, M. 2013. Noninvasive Insulin Delivery: The Great Potential of Cell-Penetrating Peptides. *Ther Deliv*, 4:315–326.

28. Kanazawa, T., Taki, H., Tanaka, K., Takashima, Y., Okada, H. 2011. Cell-Penetrating Peptide-Modified Block Copolymer Micelles Promote Direct Brain Delivery Via Intranasal Administration. *Pharm Res*, 28:2130–2139.
29. Kanemaru, Y., Momiki, Y., Matsuura, S., Horikawa, T., Gohda, J., Inoue, J., Okamoto, Y., Fujita, M., Otsuka, M. 2011. An Artificial Copper Complex Incorporating A Cell-Penetrating Peptide Inhibits Nuclear Factor-Kb (NF-Kb) Activation. *Chem Pharm Bull*, 59:1555–1558.
30. Kardani, K., Milani, A., Samaneh, H., Bolhassani, S., Bolhassani, A. 2019. Cell Penetrating Peptides: The Potent Multi-cargo Intracellular Carriers. *EXPERT OPINION ON DRUG DELIVERY*, 16(11):1217-1258.
31. Khandia, R., Munjal, A., Kumar, A., Singh, G., Karthik, K., Dhama, K. 2017. Cell Penetrating Peptides: Biomedical/Therapeutic Applications with Emphasis as Promising Futuristic Hope for Treating Cancer. *Int J Pharmacol*, 13:677-689.
32. Kinyanjui, MW., Fixman, ED. 2008. Cell-Penetrating Peptides and Proteins: New Inhibitors of Allergic Airways Disease. *Can J Physiol Pharmacol*, 86:1–7.
33. Koren, E., Torchilin, PV. 2012. Cell-Penetrating Peptides: Breaking Through to the Other Side. *Trends in molecular medicine*, 18:385-393.
34. Lecher, JC., Nowak, SJ., Mcmurry, JL. 2017. Breaking In and Busting Out: Cell-Penetrating Peptides and the Endosomal Escape Problem. *Biomol Concepts*, 8:131-141.
35. Lee, HS., Moroz, E., Castagner, B., Leroux, CJ. 2014. Activatable Cell Penetrating Peptide– Peptide Nucleic Acid Conjugate via Reduction of Azobenzene PEG Chains. *J Am Chem Soc*, 136:12868–12871.
36. Lindgren, M., Rosenthal-Aizman, K., Saar, K., Eiríksdóttir, E., Jiang, Y., Sassian, M., Ostlund, P., Hällbrink, M., Langel, U. 2006. Overcoming Methotrexate Resistance in Breast Cancer Tumour Cells by the Use of a New Cell-Penetrating Peptide. *Biochem Pharmacol*, 71:416–425.
37. Ma, S., Schroeder, B., Sun, C., Loufakis, DN., Cao, Z., Sriranganathan, N., Lu, C. 2014. Electroporation-Based Delivery of Cell-Penetrating Peptide Conjugates of Peptide Nucleic Acids for Antisense Inhibition of Intracellular Bacteria. *Integr Biol (Camb)*, 6:973–978.
38. Madani, F., Lindberg, s., Langel, Ü., Futaki, S., Gräslund, A. 2011. Mechanisms of Cellular Uptake of Cell-Penetrating Peptides. *J Biophys*, 2011:414729-414739.
39. Matsuzaki, K., Murase, O., Fujii, N., Miyajima, K. 1996. An Antimicrobial Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation. *Biochemistry*, 35:11361-11368.
40. Milleti, F. 2012. Cell-Penetrating Peptides: Classes, Origin, and Current Landscape. *Drug Discov Today*, 17:850-860.
41. Mussbach, F., Pietrucha, R., Schaefer, B., Reissmann, S. 2011. Internalization of Nucleoside Phosphates into Live Cells by Complex Formation with Different Cell Penetrating Peptides and JBS-Nucleoducin. *Methods Mol. Biol*, 683:375–389.
42. Nitin, N., Laconte, WEL., Zurkiya, O., Hu, X., Bao, G. 2004. Functionalization and Peptide-Based Delivery of Magnetic Nanoparticles as an Intracellular MRI Contrast Agent. *J Biol Inorg Chem*, 9:706–712.

43. Rahmat, D., Khan, MI., Shahnaz, G., Sakloetsakun, D., Perera, G., Bernkop-Schnürch, A. 2012. Synergistic Effects of Conjugating Cell Penetrating Peptides and Thiomers on Non-Viral Transfection Efficiency. *Biomaterials*, 33:2321–2326.
44. Reissmann, S. 2014. Cell Penetration: Scope and Limitations by the Application of Cell-Penetrating Peptides. *J Pept Sci*, 20**:**760-784.
45. Rostami, B., Irani, S., Bolhassani, A. 2019. Gene and Protein Delivery Using Four Cell Penetrating Peptides for HIV-1 Vaccine Development. *IUBMB Life*, 71(10):1619–1633.
46. Sawant, RR., Torchilin, VP. 2009. Enhanced Cytotoxicity of Tatp-Bearing Paclitaxel-Loaded Micelles In Vitro and In Vivo. *Int J Pharm*, 374:114–118.
47. Schlenk, F., Grund, S., Fischer, D. 2013. Recent Developments and Perspectives on Gene Therapy Using Synthetic Vectors. *Therapeutic Del*, 4:95–113.
48. Snyder, EL., Dowdy, SF. 2004. Cell Penetrating Peptides in Drug Delivery. *Pharm Res*, 21:389-393.
49. Stewart, KM., Horton, KL., Kelley, SO. 2008. Cell-Penetrating Peptides as Delivery Vehicles for Biology and Medicine. *Org Biomol Chem*, 6:2242-2255.
50. Torchilin, PV., Rammohan, R., Weissig, V., Levchenko, ST. 2001. TAT Peptide on the Surface of Liposomes Affords Their Efficient Intracellular Delivery Even at Low Temperature and in the Presence of Metabolic Inhibitors. *Proc Natl Acad Sci.U.S.A*, 98:8786-8791.
51. Vousden, KH., Lu, X. 2002. Live Or Let Die: The Cell's Response to P53. *Nat Rev Cancer*, 2:594-604.

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING METHOD FOR THE SIMULTANEOUS ESTIMATION OF ABACAVIR SULFATE, LAMIVUDINE AND DOLUTEGRAVIR SODIUM IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

Gorja Ashok[1] and Jahnavi Bandla[*2]

[1]Department of Pharmaceutical Analysis & QA, Faculty of Pharmacy, Gokaraju Rangaraju College of Pharmacy, Bachupally-500090, Hyderabad, Telangana, India

[2]Department of Pharmaceutical Analysis & QA, Faculty of Pharmacy, Vishnu Institute of Pharmaceutical Education and Research, Bachupally-500090, Hyderabad, Telangana, India

*Corresponding author E-mail: jahnavi.bandla@gmail.com

Abstract:

A simple, rapid, specific, stability indicating method was developed and validated for the simultaneous estimation of Abacavir sulfate, Lamivudine and Dolutegravir sodium in pharmaceutical dosage form using RP-HPLC. The chromatographic separation was done using BDS column of dimensions 250mm x 4.6mm, 5µ particle size with mobile phase consisting of potassium dihydrogen phosphate buffer and acetonitrile in the ratio 45:55%v/v run on an isocratic mode of flow rate 1.0ml/min. The column oven temperature was maintained at 30ºC. The detection was done at a wavelength of 240nm. The developed method was validated in accordance with ICH guidelines, evaluating accuracy, precision, ruggedness, robustness, LOD, LOQ, stability parameters and found to be within the limits. The method obeys Beer's law in the concentration range of 150µg/ml – 900µg/ml for Abacavir, 75µg/ml – 450µg/ml for Lamivudine and 12.5µg/ml – 75µg/ml for Dolutegravir with correlation coefficients of 0.9999, 0.9996 and 0.9999 for the three drugs respectively. Forced degradation studies were conducted by exposing the standard drug solution to the various stressed conditions such as acidic, basic, oxidative, thermal, neutral and photolytic conditions. The net degradation for the drugs was found to be within the limits.

Keywords: Abacavir sulfate, Lamivudine, Dolutegravir sodium, RP-HPLC, Stability indicating method, Method development, Validation

Introduction:

Abacavir sulfate [1, 2] (Figure 1A) is chemically designated as bis([(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-en-1-yl]methanol); sulfuric acid. It is white to off-white solid, freely soluble in water and methanol. It has pKa values of 5.77 and 15.41. It acts as antiretroviral drug by inhibiting nucleoside reverse transcriptase, and hence used for the treatment of HIV/ AIDS. Lamivudine [3, 4] (Figure 1B) is chemically designated as 4-Amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. It is white or almost white powder, soluble in water, sparingly soluble in methanol and practically insoluble in acetone. It has a pKa value of 14.29. It acts as antiretroviral drug by inhibiting nucleoside reverse transcriptase, and hence used for the treatment of HIV / AIDS and chronic Hepatitis B at low dose. Dolutegravir sodium [5, 6] (Figure 1C) is chemically designated as (4R,12aS)-N-[(2,4-Difluorophenyl)methyl]-3,4,6,8,12,12a-hexahydro-7-hydroxy-4-methyl-6,8-dioxo-2H-

pyrido[1′,2′:4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide sodium salt. It is white to light yellow powder, slightly soluble in water. It has a pKa value 10.1. It acts antiretroviral drug by inhibiting HIV integrase, and hence used for the treatment of HIV / AIDS. Literature survey [7-13] reveals that there are only few methods developed for the simultaneous estimation of Abacavir, Lamivudine and Dolutegravir in pharmaceutical dosage forms. The present study aimed to develop and validate the stability indicating method for the simultaneous determination of Abacavir, Lamivudine and Dolutegravir in pharmaceutical dosage form by RP-HPLC.

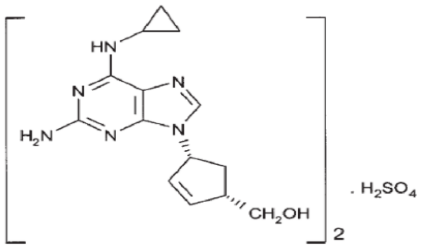


Figure 1A: Chemical structure of Abacavir sulfate

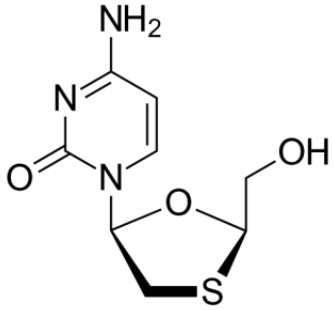


Figure 1B: Chemical structure of Lamivudine

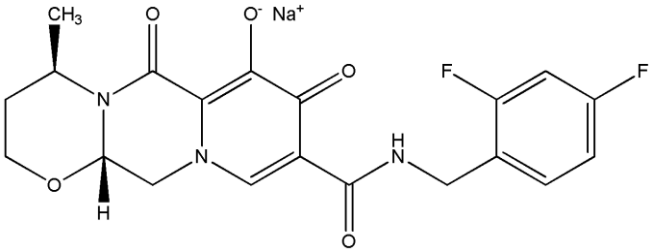


Figure 1C: Chemical structure of Dolutegravir sodium

Material and Methods:

Chemicals and Reagents: Abacavir sulfate, Lamivudine and Dolutegravir sodium working standards were supplied by Spectrum labs, Hyderabad, India as gift samples. The tablets were purchased from local pharmacy. All the chemicals used for the development of method were of AR grade. All the solvents used for method development were of HPLC grade.

Analytical instruments and Chromatographic conditions: The separation of drugs was done using HPLC Waters 2998 model equipped with an autosampler, Photo diode array detector with empower 2 software. Column used for separation was BDS (250mm x 4.6 mm, 5µ) with mobile phase consisting of Potassium dihydrogen phosphate and acetonitrile in the ratio 45:55% v/v on isocratic mode at 1.0ml/min flow rate. The detection was done at 240nm and column oven temperature was maintained at 30ºC. The other instruments used were pH meter (EI), Digital Balance (Infra Instruments), Ultrasonic Bath (Wadegati), Hot air oven (Cisco).

Preparation of mobile phase: Transfer 1.36g of potassium dihydrogen phosphate in to a 1000mL volumetric flask; add about 100ml of milli-Q water and mix. Finally make volume up to the mark with milli-Q water. Adjust the pH to 5.8.

Mixture of above phosphate buffer and Acetonitrile in the ratio 45:55 (%v/v) respectively was used as mobile phase.

Preparation of standard and sample solutions: Dissolve 120mg of Abacavir working standard, 60mg of Lamivudine and 10mg of Dolutegravir working standard in 100ml of diluent. Dilute 1ml of the above stock solution to 10ml with diluent.

20 tablets (Triumeq) were weighed accurately and the average weight was calculated. An amount equivalent to 120mg of Abacavir was weighed and dissolved in 100ml of diluent using sonicator for 30min with intermediate shaking. The above solution was filtered using HPLC filters. 1mL of the above solution was pipette into 10mL volumetric flask and made up with diluent.

Method Validation [14, 15]:

The developed method was validated as per ICH guidelines. The following parameters were validated; accuracy, precision, linearity, specificity, ruggedness, robustness and stability. Forced degradation studies [16] were also conducted by exposing the drugs solution to various conditions such as acidic, basic, peroxide, thermal, neutral and photolytic conditions.

Results and Discussion:

For the development of method for the simultaneous determination of Abacavir, Lamivudine and Dolutegravir, initially various mobile phase compositions and columns were tried for eluting the drugs with good peaks and parameters. Potassium dihydrogen phosphate and acetonitrile in the ratio 45:55%v/v at flow rate 1.0ml/min was selected as mobile phase. BDS (250mm x 4.6mm, 5µ) column was selected as stationary phase for separation of drugs. The column oven temperature was maintained at 30ºC. The detection wavelength was selected by scanning the drugs solution in the UV range of 400nm – 200nm and was found to be 240nm as shown in figure 2.

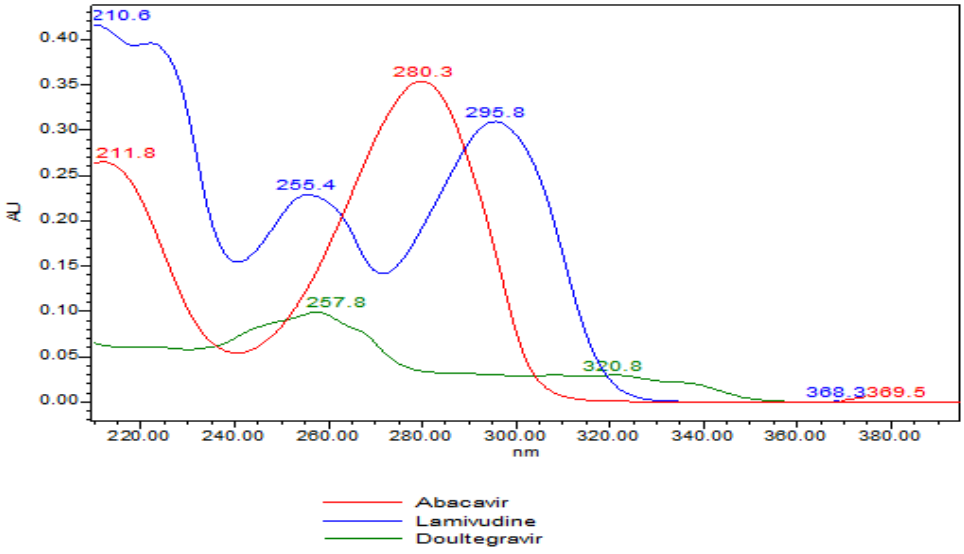


Figure 2: Overlain UV Spectrum

HPLC Method development trails

A) Trial I:

Column : BDS (250mm x 4.6 mm, 5µ)
Mobile section : Water: Methanol (50:50)
Diluent : Water: Acetonitrile (50:50)
Mode : Isocratic
Rate of flow : 1mL/minute
Temperature in column : 30ºC
Volume of injection : 10µL
Detection Wave Length : 240nm
USP Plate Count : 6294 (Abacavir), 10944 (Lamivudine)
USP Tailing : 1.3 (Abacavir), 1.2 (Lamivudine)
USP Resolution : 13.0

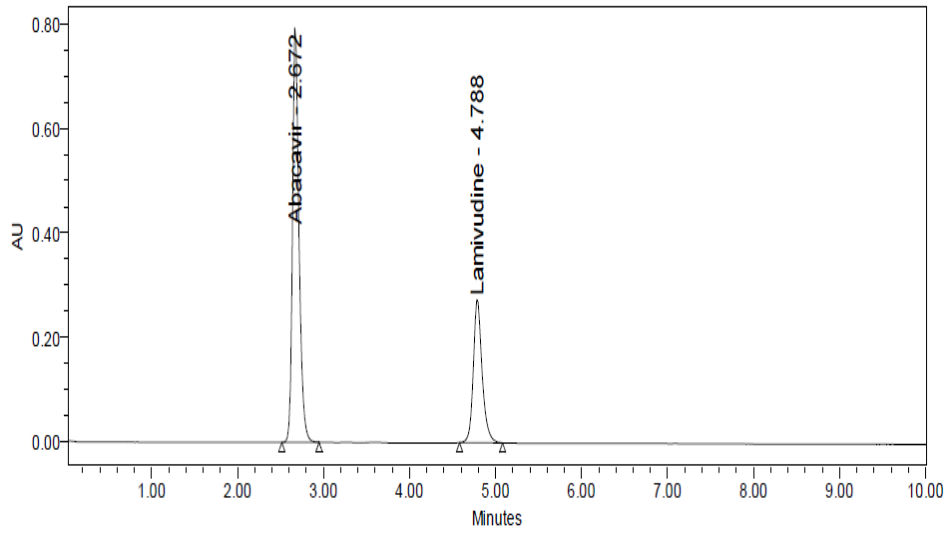


Figure 3.1: HPLC trail 1 chromatogram of Abacavir, Lamivudine and Dolutegravir

Observation: Only two peaks were eluted, so proceeded to next trail.

B) Trial II:

Column : BDS (250mm x 4.6 mm, 5µ)
Mobile phase : Water: Acetonitrile (50:50)
Diluent : Water: Acetonitrile (50:50)
Mode : Isocratic
Flow Rate : 1.0mL/min
Column Temperature : 30ºC
Injection Volume : 10µL
Wave Length : 240nm
USP Plate Count : 5314 (Abacavir), 13735 (Lamivudine)
USP Tailing : 1.2 (Abacavir), 1.2 (Lamivudine)
USP Resolution : 11.4

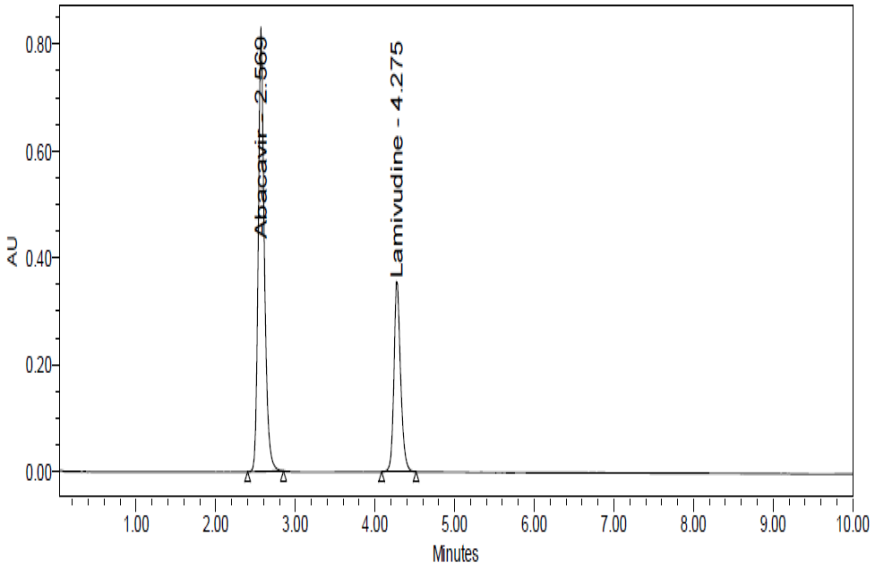


Figure 3.2: HPLC trail 2 chromatogram of Abacavir, Lamivudine and Dolutegravir

Observation: Only two peaks were eluted, so proceeded to next trail

C) Trial III:

Column : BDS (250mm x 4.6 mm, 5µ)
Mobile phase : Perchloric acid (0.01N): Acetonitrile (50:50)
Diluent : Water: Acetonitrile (50:50)
Mode : Isocratic
Flow Rate : 1.0mL/min
Column Temperature : 30ºC
Injection Volume : 10µL
Wave Length : 240nm
USP Plate Count : 6816 (Abacavir), 7489 (Lamivudine) & 10137 (Dolutegravir)
USP Tailing : 1.5 (Abacavir), 1.1 (Lamivudine) & 1.2 (Dolutegravir)
USP Resolution : 6.9 & 2.1

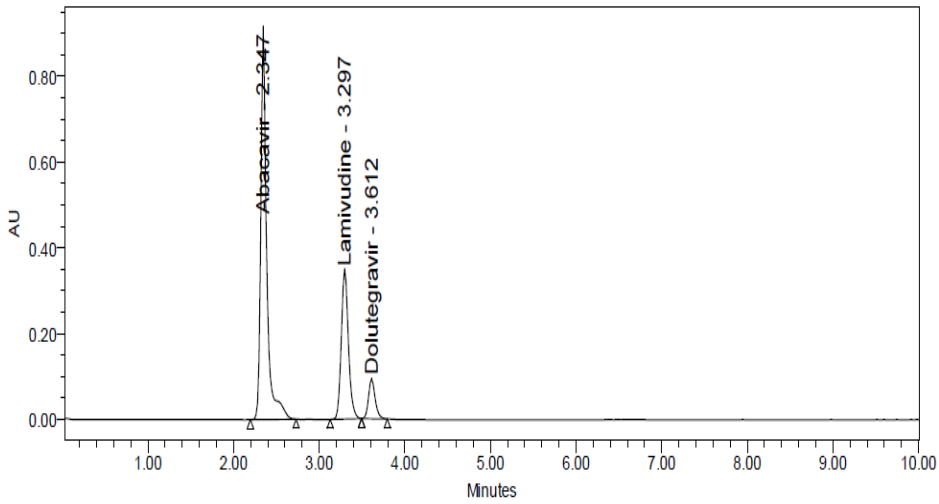


Figure 3.3: HPLC trail 3 chromatogram of Abacavir, Lamivudine and Dolutegravir

Observation: Three peaks were eluted, but peaks shape was not good, so preceded to next trail.

D) Trial IV:

Column : BDS (250mm x 4.6 mm, 5µ)
Mobile phase : Perchloric acid (0.01N): Acetonitrile (60:40)
Diluent : Water: Acetonitrile (50:50)
Mode : Isocratic
Flow Rate : 1.0mL/min
Column Temperature : 30ºC
Injection Volume : 10µL
Wave Length : 240nm
Plate Count of USP : 6288 (Abacavir), 9413 (Lamivudine) & 11792 (Dolutegravir)
USP Tailing : 1.1 (Abacavir), 1.2 (Lamivudine) & 1.3 (Dolutegravir)
USP Resolution : 7.6 & 2.0

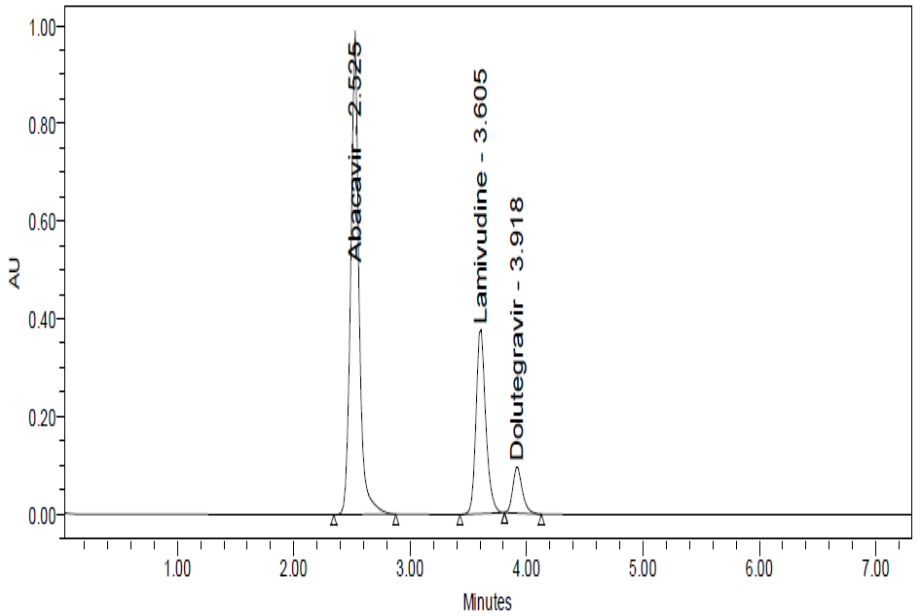


Figure 3.4: HPLC trail 4 chromatogram of Abacavir, Lamivudine and Dolutegravir

Observation: Three peaks were eluted, but resolution between last two peaks was not good, so preceded to next trail.

E) Trial V:

Column : BDS (250mm x 4.6 mm, 5µ)
Mobile phase : KH_2PO_4: Acetonitrile (50:50)
Diluent : Water: Acetonitrile (50:50)
Mode : Isocratic
Flow Rate : 1.0mL/min
Column Temperature : 30ºC
Injection Volume : 10µL
Wave Length : 240nm
USP Plate Count : 5439 (Abacavir), 9775 (Lamivudine) & 10759 (Dolutegravir)
USP Tailing : 1.3 (Abacavir), 1.2 (Lamivudine) & 1.2 (Dolutegravir)
USP Resolution : 9.0 & 5.1

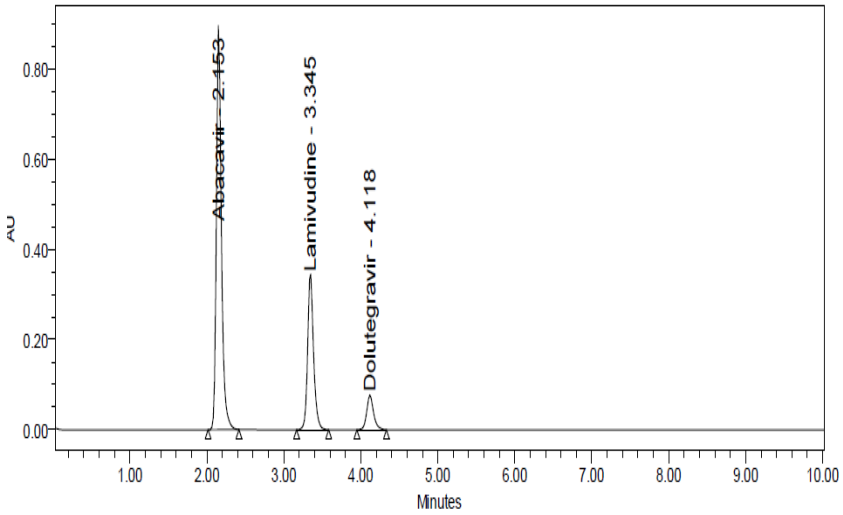


Figure 3.5: HPLC trail 5 chromatogram of Abacavir, Lamivudine and Dolutegravir

Observation: Three peaks were eluted, but retention time was more, so preceded to next trail.

F) Trial VI:

Column	: BDS (250mm x 4.6 mm, 5µ)
Mobile phase	: KH_2PO_4: Acetonitrile (45:55)
Diluent	: Water: Acetonitrile (50:50)
Mode	: Isocratic
Flow Rate	: 1.0mL/min
Column Temperature	: 30ºC
Injection Volume	: 10µL
Wave Length	: 240nm
Plate Count of USP	: 6022 (Abacavir), 8207 (Lamivudine) & 9148 (Dolutegravir)
USP Tailing	: 1.1 (Abacavir), 1.2 (Lamivudine) & 1.1 (Dolutegravir)
USP Resolution	: 6.9 & 3.9

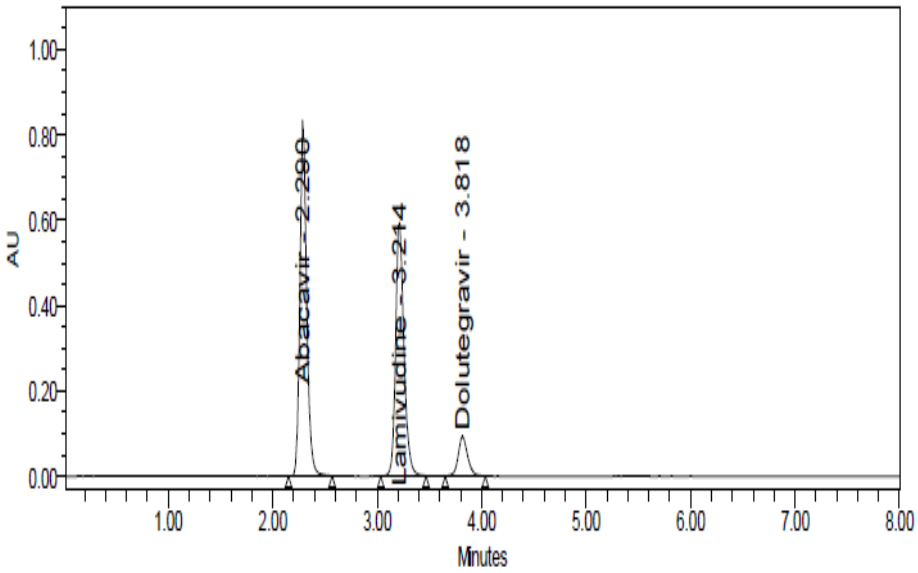


Figure 3.6: **HPLC trail 6 chromatogram of Abacavir, Lamivudine and Dolutegravir**

Observation: Three peaks were eluted with good system suitability parameters and with less retention time.

The standard, sample and blank solutions were prepared and injected into the chromatographic system. The system suitability parameters were noted and the chromatograms were shown in figure 4A, 4B and 4C respectively.

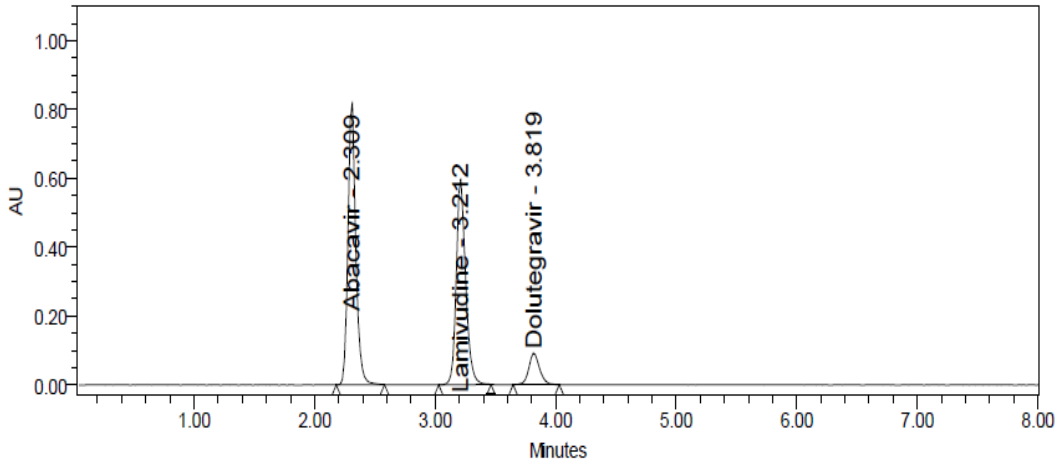


Figure 4A: Standard Chromatogram

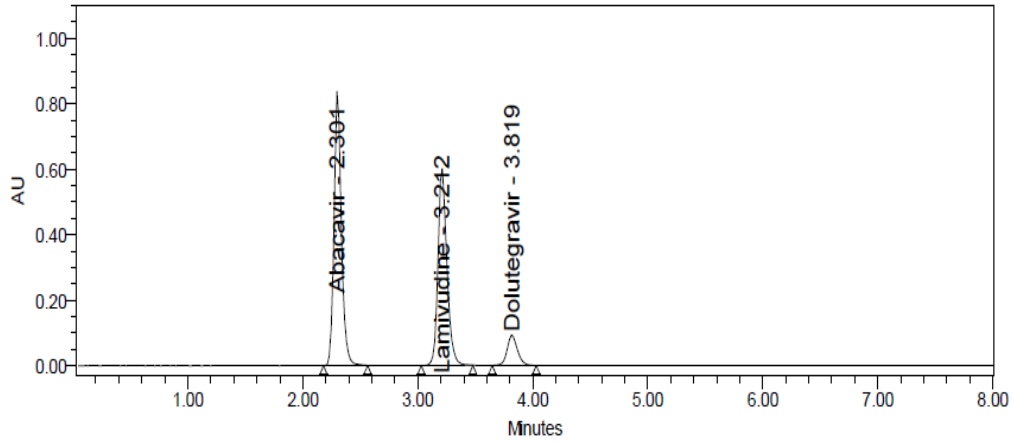


Figure 4B: Sample Chromatogram

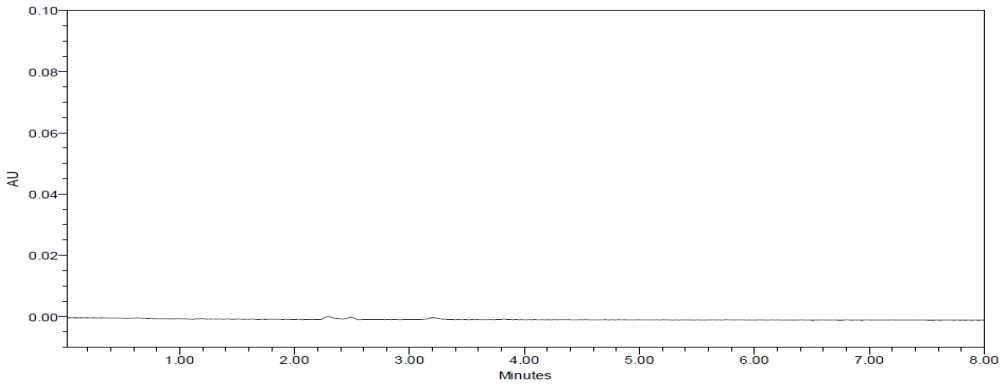


Figure 4C: Blank Chromatogram

The specificity of the method was determined by comparing with placebo and observed for any interference. No interference was observed at retention times of Abacavir, Lamivudine

and Dolutegravir peaks when compared with placebo solution. The placebo chromatogram was shown in figure 5.

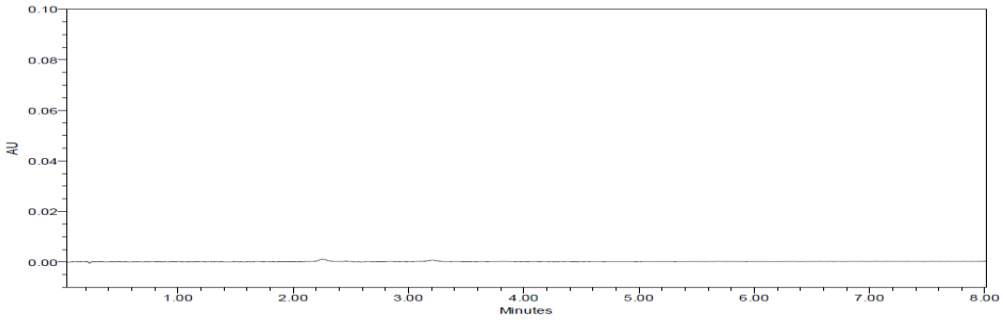
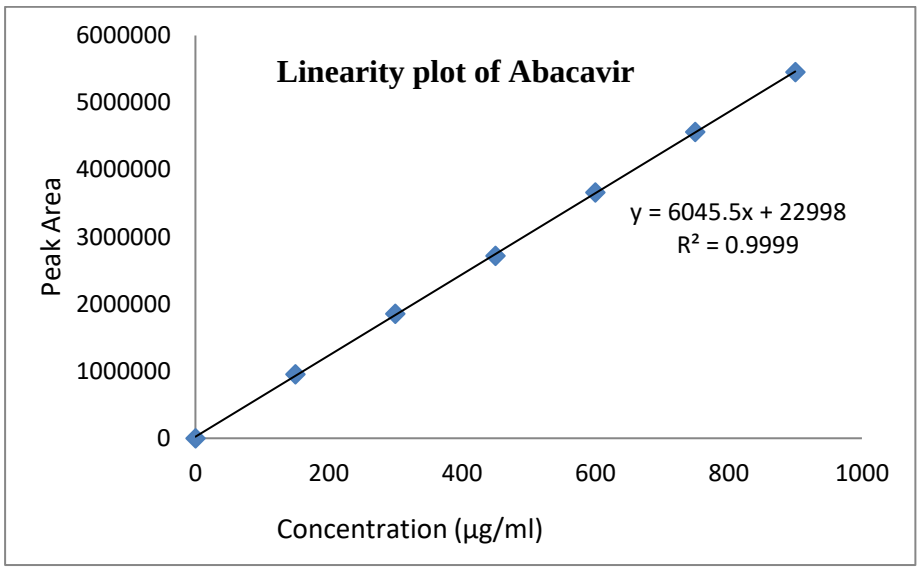
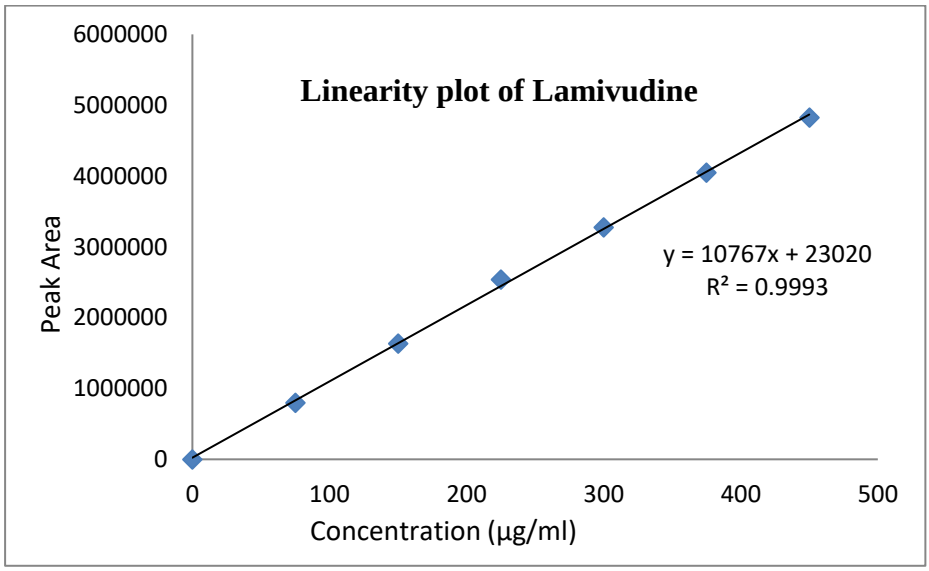


Figure 5: Placebo Chromatogram

The method obeys Beer's law in the concentration range of 150µg/ml – 900µg/ml for Abacavir, 75µg/ml – 450µg/ml for Lamivudine and 12.5µg/ml – 75µg/ml for Dolutegravir with correlation coefficient of 0.9999, 0.9996 and 0.9999 for Abacavir, Lamivudine and Dolutegravir respectively, indicates that the method is linear. The linearity plots were shown in figure 6 and results were summarized in table 1.





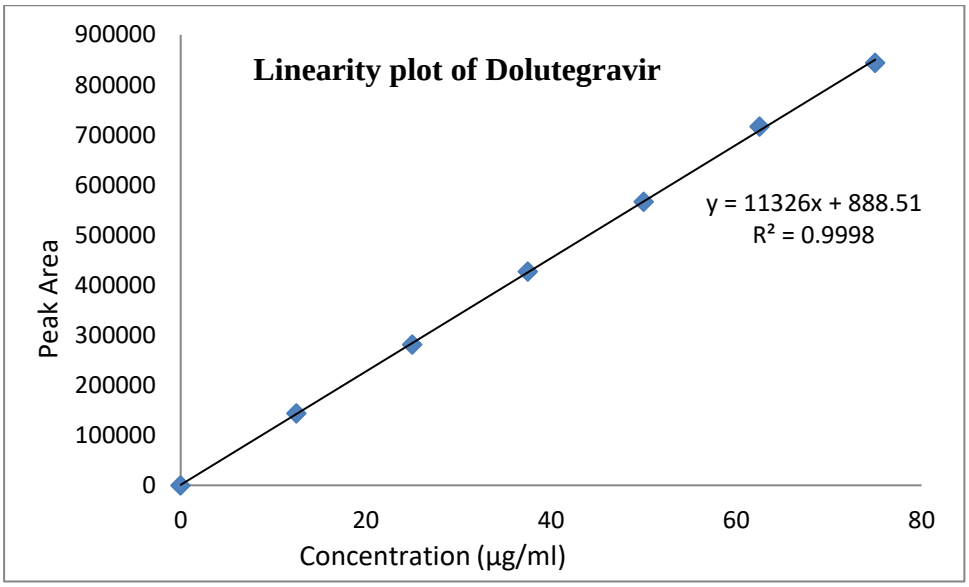


Figure 6: Linearity plot of A) Abacavir B) Lamivudine and C) Dolutegravir

Table 1: Linearity results

Parameter	Abacavir	Lamivudine	Dolutegravir
Linearity (µg/ml)	150 – 900	75 – 450	12.5 – 75.0
Regression equation, y=mx+c	y = 6045.5x + 22998	y = 10767x + 23020	y = 11326x + 888.51
Slope, m	6045.5	10767	11326
Y-intercept, c	22998	23020	888.51
Regression coefficient, r^2	0.9999	0.9993	0.9998
Correlation coefficient, r	0.9999	0.9996	0.9999

The % recovery for Abacavir, Lamivudine and Dolutegravir were found to be 99.80% - 100.21%, 99.36% - 99.79% and 99.80% - 100.10% respectively. The % RSD for Abacavir, Lamivudine and Dolutegravir were found to be 0.2, 0.5 and 0.2 respectively as the results were within the limits indicating the method to be accurate and precise. The Limit of Detection (LOD) for Abacavir, Lamivudine and Dolutegravir were found to be 1.69µg/ml, 1.23µg/ml and 0.04µg/ml respectively. The Limit of Quantitation (LOQ) for Abacavir, Lamivudine and Dolutegravir were found to be 5.11µg/ml, 3.74µg/ml and 0.11µg/ml respectively. The method was found to be rugged, robust and stable in solution for 24hours. The results are summarized in table 2.

Table 2: System Suitability and Validation Parameter Results

Parameter	Abacavir	Lamivudine	Dolutegravir
Specificity	Specific	Specific	Specific
Precision (% RSD)	0.2	0.5	0.2
Accuracy (% Recovery)	99.80%-100.21%	99.36%-99.79%	99.80%-100.10%
Linearity range (µg/ml)	150 – 900	75 – 400	12.5 – 75.0

Correlation coefficient**,** r	0.9999	0.9996	0.9999
Limit of Detection (µg/ml)	1.69	1.23	0.04
Limit of Quantitation (µg/ml)	5.11	3.74	0.11
Ruggedness (%RSD)	0.2	0.7	0.4
Robustness	Robust	Robust	Robust
Solution stability	Stable	Stable	Stable
USP Plate Count	6066	8154	9106
USP Tailing Factor	1.17	1.20	1.12
USP Resolution		6.8	3.9

Forced degradation studies were conducted and the net degradation was found to be within the limits indicating that the drugs are stable at various stress conditions. The results were summarized in table 3 and chromatograms were shown in figure 7.

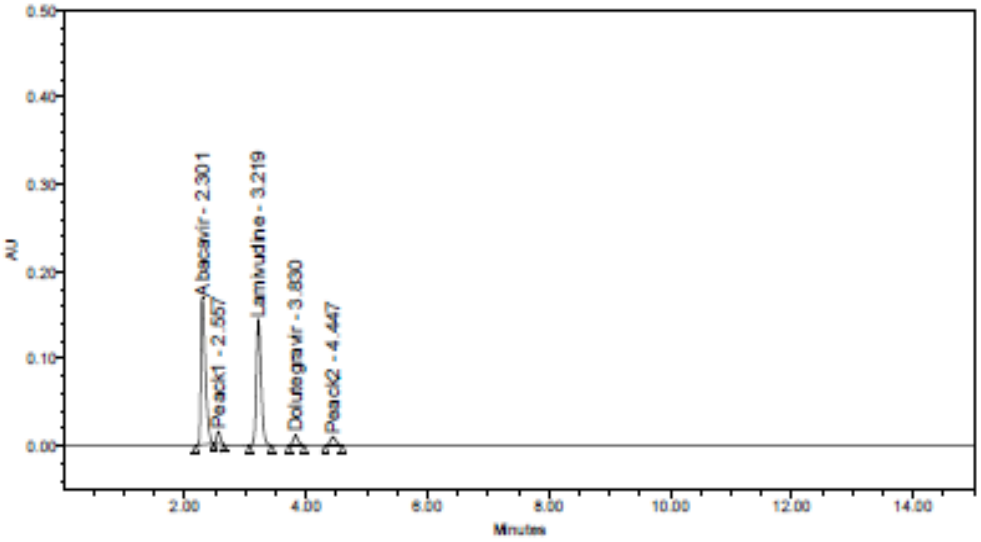


Figure 7A: Acid degradation chromatogram

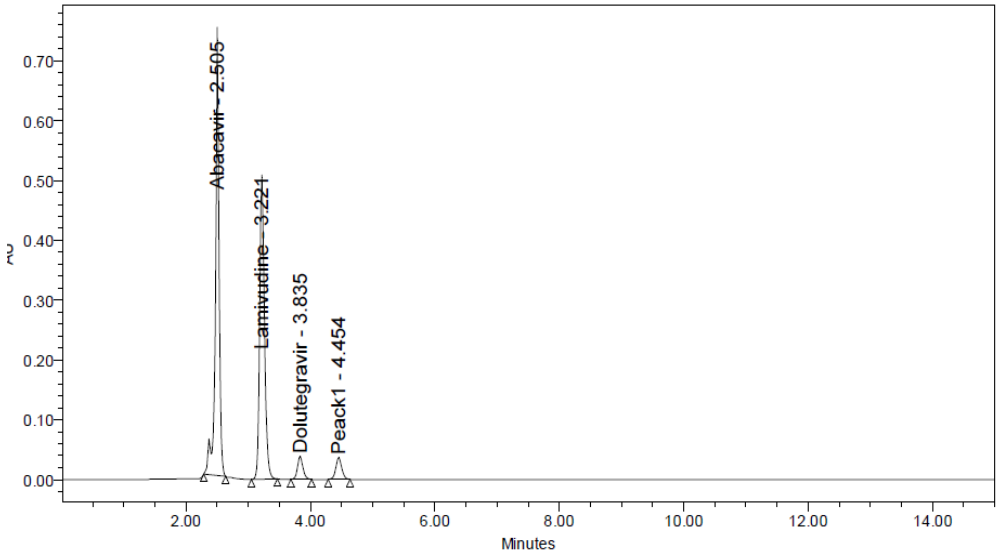


Figure 7B: Base degradation chromatogram

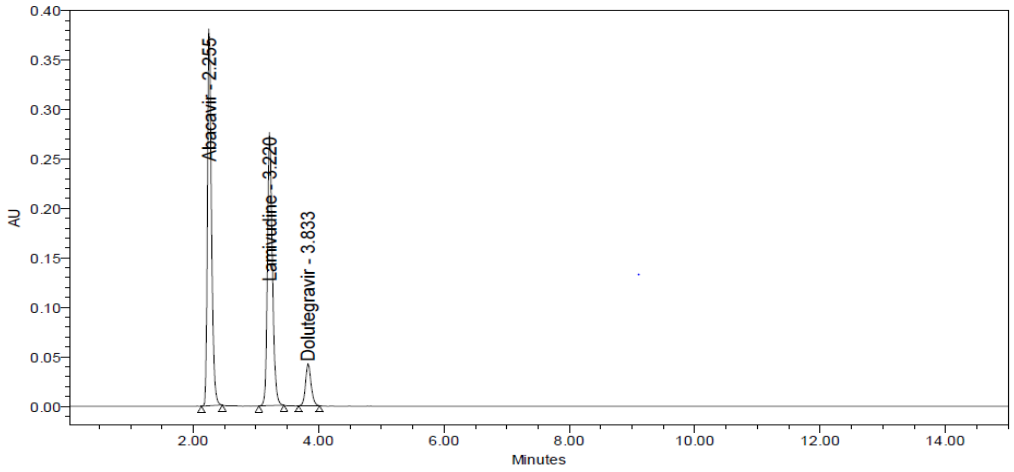


Figure 7C: Peroxide degradation chromatogram

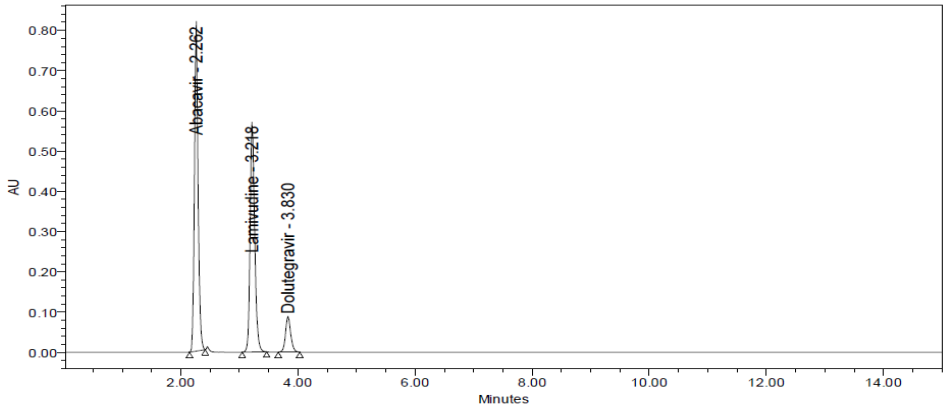


Figure 7D: Water stress study chromatogram

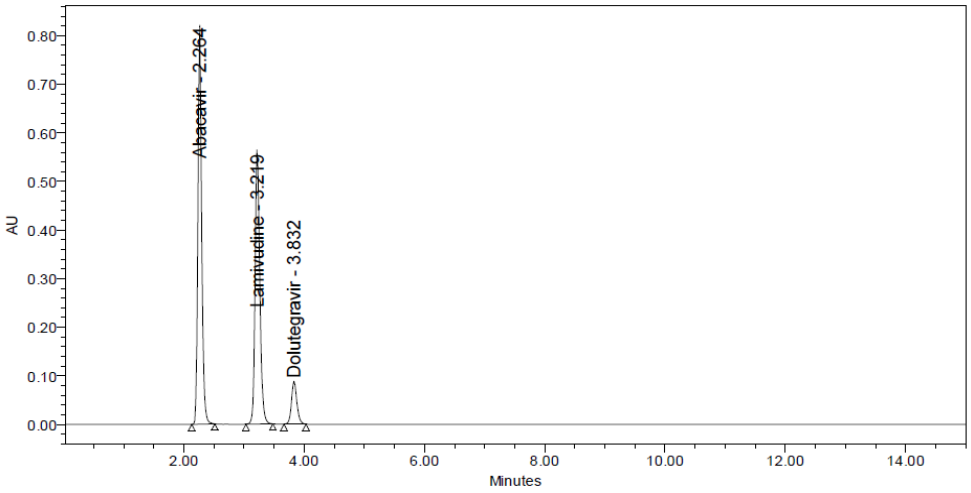


Figure 7E: Photo stability degradation chromatogram

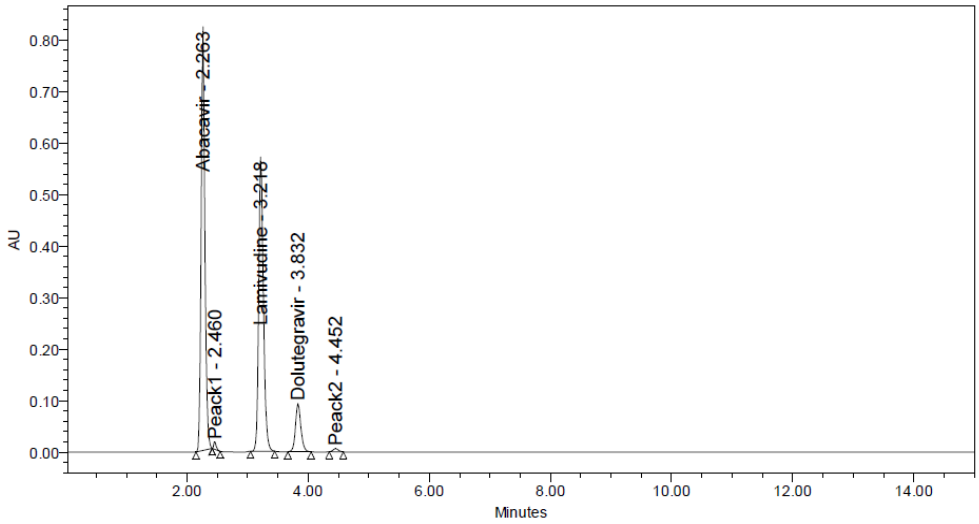


Figure 7F: Dry heat study chromatogram

Table 3: Forced degradation studies results

Drug	Parameters	Stress Condition					
		Acidic	Basic	Oxidative	Photolytic	Neutral	Dry heat
Abacavir	% Assay	95.63	97.20	98.24	99.59	99.49	99.60
	Purity Angle	0.280	0.430	0.168	0.272	0.328	0.384
	Purity Threshold	0.306	0.725	0.289	1.023	0.958	1.000
	% Degradation	4.37	2.80	1.76	0.41	0.51	0.40
Lamivudine	% Assay	95.41	97.55	98.18	98.44	99.06	98.26
	Purity Angle	0.209	0.095	0.134	0.094	0.099	0.142
	Purity Threshold	0.319	0.281	0.288	0.282	0.280	0.280
	% Degradation	4.59	2.45	1.82	1.56	0.94	1.74
Dolutegravir	% Assay	95.56	97.30	98.85	99.52	99.18	99.51
	Purity Angle	1.181	0.436	0.384	0.195	0.189	0.197
	Purity Threshold	1.589	0.668	0.625	0.410	0.404	0.404
	% Degradation	4.44	2.70	1.15	0.48	0.82	0.49
% Area of degradation Peak		6.88	3.94	-	-	-	1.10

Conclusion:

A specific, accurate stability indicating method was developed for the simultaneous determination of Abacavir, Lamivudine and Dolutegravir in pharmaceutical dosage form using RP-HPLC. The developed method is validated as per ICH guidelines and found to be accurate, specific, precise, linear, rugged, and robust and stable in solution. Forced degradation studies confirmed that the drugs are stable at high concentrations of various stress conditions.

The proposed method is used for the simultaneous estimation of Abacavir, Lamivudine and Dolutegravir in routine and quality control analysis of pharmaceutical formulations.

Acknowledgements:

The author thanks Hetero Drugs Pvt. Ltd., Hyderabad for supplying the drugs as gift samples. The author also thanks Spectrum Labs, Hyderabad for providing the facilities to carry out this research work.

References:

1. Indian Pharmacopoeia, Government of India. Ministry of health and Family welfare, the controller of publication, New Delhi. (2007). Abacavir, Volume-II, 1278-1279.
2. Pradeep Nagisetty, S.M. Shanta Kumar, Putta Rajesh Kumar. (2010). Analytical Method Development and Validation of Anti-HIV Drug Abacavir Sulphate. Journal of Young Pharmacists, 2(4), 417-419.
3. British Pharmacopoeia, H.M. Stationary office London. (2008). Lamivudine, Volume-II, 2032-2034.
4. Deepali G, Elvis M. (2010). UV Spectrophotometric Method for Assay of the Anti-Retroviral Agent Lamivudine in Active Pharmaceutical Ingredient and in its Tablet Formulation. Journal of Young Pharmacists, 2(4), 417-419.
5. International Pharmacopoeia Monograph on Dolutegravir, World Health Organization, Geneva-27 Switzerland, The World Health Organization. (2005). Working document QAS/05.145, 1-7.
6. Bhavar Girija Balasaheb, Aher Kiran Balasaheb, Thorat Ravindra Subhash, Kakad sachin Jijabapu, Pekamwar Sanjay Sudhakar. (2015). Development and Validation of UV Spectrophotometric Method for Estimation of Dolutegravir Sodium in Tablet Dosage Form. Malaysian Journal of Analytical Sciences, 19(6), 1156 – 1163.
7. Narottam Pal, Avanapu Srinivasa Rao and Pigilli Ravikumar. (2016). Simultaneous HPLC Method Development and Validation for Estimation of Lamivudine, Abacavir and Dolutegravir in Combined Dosage Form with their Stability Studies. Asian Journal of Chemistry, 28(2), 273-276.
8. M.Monica and D.Gowri Sankar. (2015). Simultaneous RP-HPLC determination of abacavir, lamivudine and dolutegravir in bulk API dosage forms. Int. J. of Pharmacy and Analytical Research, 4(3), 391-398.
9. SK. Shaista Fatima, P. Nagaraju, V. Mounika, G. Indira Priyadarshini and V. Vasu Naik. (2017). stability-indicating method development and validation of RP-HPLC method for simultaneous estimation of Lamivudine, Abacavir, Dolutegravir in pharmaceutical dosage forms. Indo American Journal of Pharmaceutical Sciences, 4(2), 359-367.
10. Sindu Priya and D. Gowri Sankar. (2016). Simultaneous stability-indicating method for the determination of abacavir, dolutegravir and lamivudine by RP-HPLC. International Journal of Pharmaceutical Sciences and Research, 7(7), 2905-2916.
11. P. Saidulu, Sk. Mastanamma. (2016). Stability Indicating Gradient RP-HPLC Method for the Simultaneous Estimation of Lamivudine, Abacavir and Dolutegravir in Bulk and their Combined Dosage Form. International Journal of Pharmaceutical Sciences Review and Research, 37(2), 249-257.
12. N. Khaleel and Sk. Abdul Rahaman. (2015). A validated stability indicating RP-HPLC method for simultaneous determination of abacavir, lamivudine and dolutegravir in bulk

and pharmaceutical dosage form. World Journal of Pharmaceutical Research, 4(07), 1453-1476

13. Bommakanti Valli Purnima, Merugu Santha Kumari, Golkonda Ramu, Tummala Vijaya Bhaskara Reddy, Dittakavi Ramachandran. (2016). Validated Reversed Phase HPLC Method for Assay and Degradation Studies of Lamivudine, Abacavir Sulphate And Dolutegravir In Combined Dosage Form. International Journal of education and applied research, 6(2), 86- 95.
14. ICH: Q2 (R1), Validation of analytical procedures: text and methodology; 2005.
15. United States Pharmacopeial Convention: The United States Pharmacopeia, USP NF 29, Validation of compendial methods. The United States Pharmacopeial Convention, 2005, 3050–3053
16. G. Ngwa. (2010). Forced degradation studies as an integral part of HPLC stability indicating method development. Drug Deliv. Technol., 10 (5), 56–59.

COUMARIN SCAFFOLD: A POTENTIAL ANTICANCER WITH DIVERSE MECHANISMS

Noel, Arshveer Singh, Pulkit Madan, Ashwani Kumar and Monika Gupta*
Amar Shaheed Baba Ajit Singh Jhujhar Singh Memorial College of Pharmacy,
Bela, Ropar, India
*Corresponding author E-mail: monikaguptaa@gmail.com

Abstract:

The most dreadful condition that poses a threat to life is cancer. The need for the development of innovative anticancer medications with a variety of mechanisms of action is driven by the multidrug-resistant malignancies and non-selectivity. One of the most popular scaffolds for the creation of potent anticancer drugs is coumarin. In light of this, medicinal chemists and drug design experts began investigating several coumarin-based derivatives in order to understand their potential to create new effective anticancer drugs. With a focus on their cellular and enzymatic mechanisms of action, the current study gives an overview of several anticancer classes built on the coumarin scaffold that have been described since 2015. Several researchers have thoroughly studied various kinds of coumarin-based anticancer drugs that work *via* various modes of action, such as adenosine receptor ligands (GPCRs): Coumarin, DNA interactive molecules (HepG2 (liver cancer), HCT116 (colon cancer), A549 (lung cancer), MCF7 (breast cancer), SGC7901 (stomach cancer), SGC7901/cis (cisplatin-resistant stomach cancer) , guanine in DNA, antimitotic agents, apoptosis inducers, carbonic anhydrase inhibitors, Hormone antagonists telomerase inhibitors and other mechanisms. In addition to being often used to treat leukaemia, prostate cancer, and renal cell carcinoma, coumarins also have the power to mitigate the negative effects of radiation. Due to its use in photochemotherapy and other cancer treatments, coumarin derivatives, both natural and synthetic, are in great demand. The anticancer properties of coumarin derivatives are excellent, and they also have little adverse effects.

Keywords: Coumarin, DNA interactive molecules, antimitotic agents, apoptosis inducers, carbonic anhydrase inhibitors, telomerase inhibitors

Introduction:

Coumarin is polyphenolic compound belonging a group of colourless and crystalline oxygenated heterocyclic compounds first isolated from the plant named *Dipteryx odorata* Wild. It belongs to Fabaceae Family known locally as “coumaroun” by Vogel in 1820. The pyran derivatives are ketonic compounds that in the form of α-pyron or γ-pyron. Secondary metabolites called benzo-α-pyron and benzo-γ-pyron occur due to condensation of pyron derivatives with benzene in plants.

A total of 800 coumarin derivative compounds that naturally found were obtained from about 600 genera of 100 families to date. Coumarin and its derivatives are frequently found in the seeds, roots and leaves of many plant species belonging to families especially Rutaceae in the Dicotyledonous class of the division of Spermatophytes. Although most natural coumarin is isolated from vascular plants, some coumarin such as novobiocin, coumermycin and aflatoxin are isolated from microbial sources.

These compounds have become of importance in recent years due to their various biological activities. Previous biological activity studies performed on coumarin derivatives revealed that these compounds have antitumor, photo chemotherapy, anti-HIV, antibacterial and antifungal, anti-inflammatory and central nervous system stimulant effects. Both natural and synthetic coumarin derivatives draw attention due to their photo chemotherapy and therapeutic applications in cancer.

Multi-target compounds are recently being searched for and these compounds are thought to be promising compounds for the treatment of several disorders, including cancer and heart failure. In this context, compounds observed from natural sources come into prominence due to their low toxicity, low drug resistance, low cost and high efficacy. Therefore, new compounds isolated from natural sources, such as plants and animals, and possible combination of these compounds with conventional chemotherapeutic agents seems to be important strategies to improve life quality, especially in cancer patients. Coumarin-based structure compounds constitute a major group of natural compounds with various pharmacological effects. These groups of compounds can be isolated from different plants, including Achillea, Artemisia and Fraxinus genera, and also, they can be synthesized through various chemical reactions. Several strategies such as maceration, reflux, ultrasonic-assisted and microwave extraction methods are used for the isolation and purification of coumarin compounds.

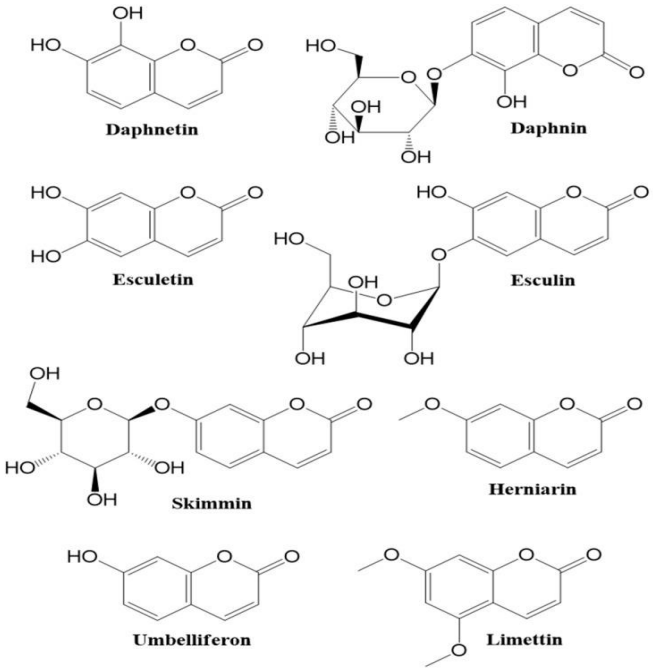


Figure 1: Structures of coumarin-based compounds

Perkin, Von Pechmann, Knoevenagel and Wittig organic reactions are some of reactions that coumarin can be synthesized. In the biosynthetic origin of coumarin, the shikimic acid pathway plays an important role. In this pathway, there are several enzymatic steps leading to occur chorismic acid, cinnamon acid, p-coumarin acid and umbelliferone. Moreover, the cytochrome P450 enzymes have a crucial role in the ortho-hydroxylation of cinnamon acid leading to occur umbelliferone, scopolamine and isofraxidin.

Cancer

Cancer, known as the abnormal division, proliferation and accumulation of cells in an organism, is one of the most common causes of disease-related deaths worldwide. It can affect a single organ as well as spread to distant organs.

Since the division and growth of cells are controlled by genes, cancer is basically a disease associated with genes. The genes on the chromosomes are tightly packed, and physical or chemical changes on these genes can directly affect the cell's function. Although DNA repair systems can ameliorate the function of the gene in case of damage, they cannot be always successful. In this case, inadequate or incorrect production of proteins as the products of genes leads to the disruption in cellular functions. Another factor that affects the function of the gene is epigenetic modifications, such as Methylation, acetylation, Phosphorylation and ribosylation, which change the function of the gene without changing its structure. These modifications can only act on a specific site, but also, they may cause of regional deletions, insertions or inversions that affect all or a large part of the chromosomes.

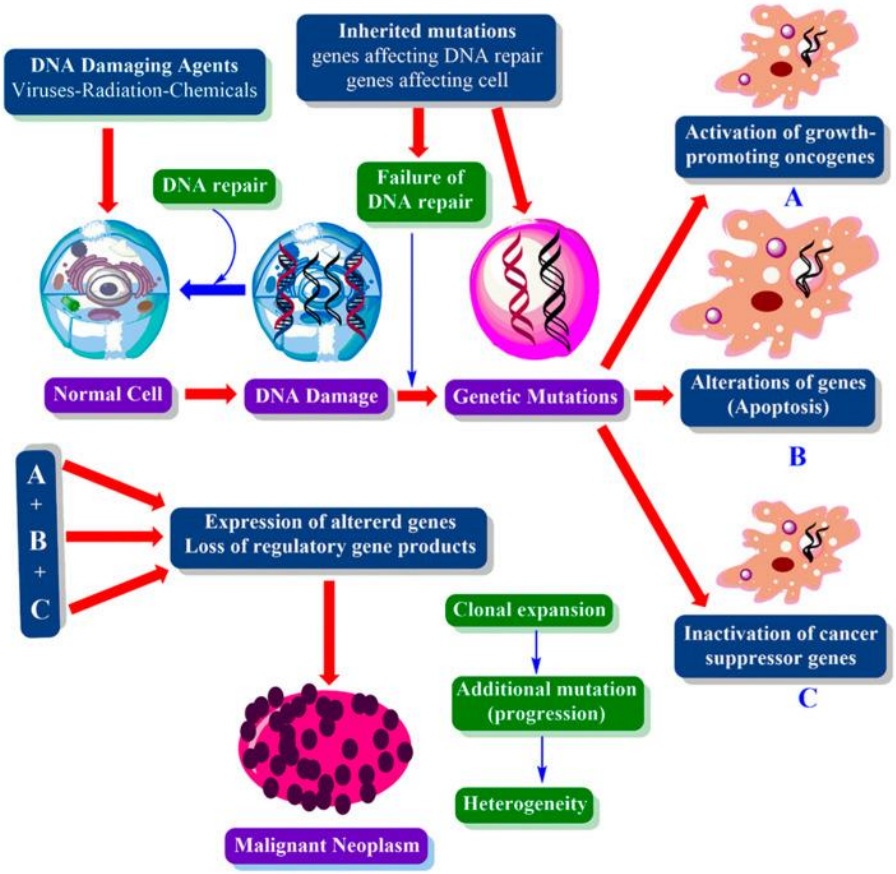


Figure 2: Cancer mechanism involving oncogenes, tumour suppressor genes and DNA repair genes

There are three gene groups that play crucial role in cancer formation oncogenestumor suppressor genes and DNA repair genes. Proto-oncogenes, which are normal genes that enable cell growth and differentiation, can be active and turn into oncogenes due to mutations, increased gene expression, gene duplications or chromosomal rearrangements. Tumour suppressor genes control cell division and proliferation, initiate DNA repair in case of damage and trigger

apoptosis if repair attempt falls down. Deletions, point mutations, epigenetic gen silencing, improper separation of chromosomes and mitotic recombination can lead to loss of control of the tumour suppressor gene, resulting in loss of control in the cell cycle and carcinogenesis. Another important group of genes are DNA repair genes that attract the necessary proteins to the site of damaged DNA, thereby restoring the function of the gene. Providing the destroying of cell's apoptotic or necrotic pathway in case of ineffective repair is another important function of DNA repair genes. However, loss of function in this important gene group is a common problem in cancer formation of the cell. One of the most known DNA repair genes is the breast cancer (BRCA) gene, which causes breast cancer due to impaired function.

Under normal conditions, when cells receive signals from the outer membrane, they grow, divide and proliferate. The signals coming from outer membrane enter into the cell, transferred to the nucleus and the process begins. Before the division, a cell checks its surroundings and checks whether there is enough nutrients and place to grow, and begins to grow if conditions are favourable. They grow until they reach the predetermined size and number and stop growing as they touch each other. In case of damage of one of the DNA or cell elements, the cells stop growing and dividing in order to move to a phase called the G0 phase which provides repairment. If the cell is repaired with the necessary arrangements in this phase, it is included in the circulation again and continues its life. However, if a cell is damaged beyond repair, apoptosis is initiated resulting in death of the cell or the immune system cells demolish the damaged cell. Thus, transferring the damaged DNA to the next generations is prevented. On the other hand, cancer cells have their own signal systems that allow abnormal growth differently from normal cells, and do not stop dividing after contact with other cells; they continue to grow and proliferate.

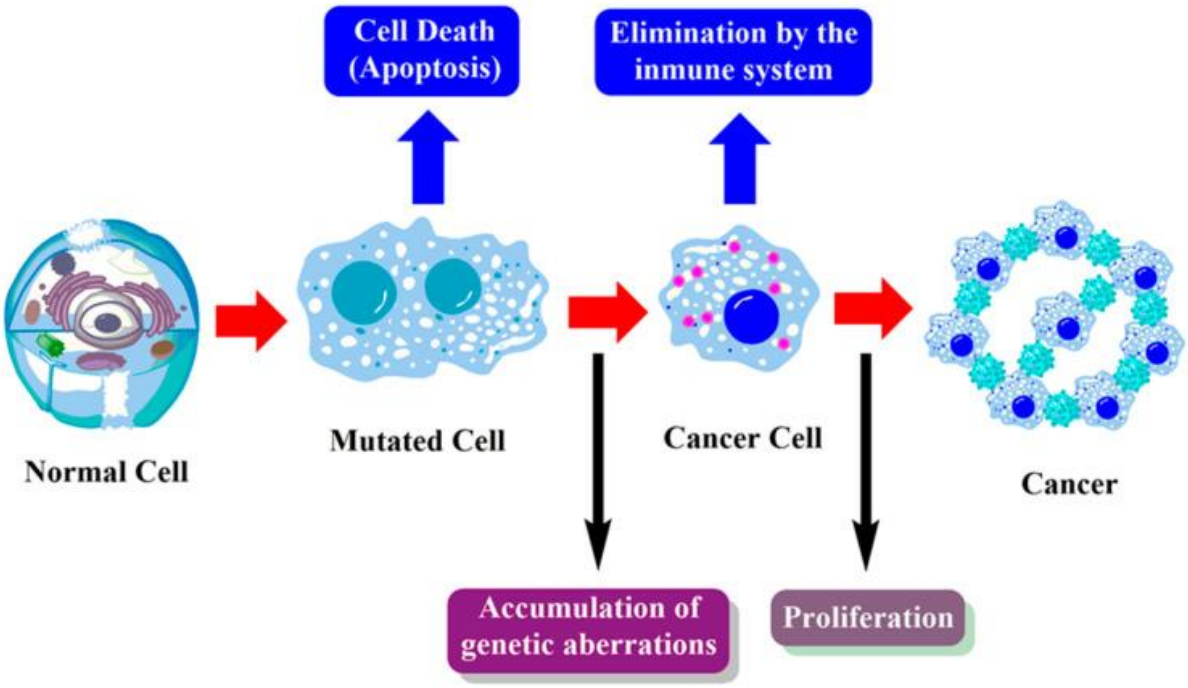


Figure 3: Mechanism of carcinogenesis

Basic mechanism of carcinogenesis

Tobacco and tobacco products, alcohol, malnutrition, obesity, viruses, exposure to ionizing rays, occupational diseases and environmental pollutants can cause cancer. Lung cancer is the most common type of cancer that causes death in both men and women. In the second place are prostate cancer in men and breast cancer in women.

Cancer treatment

Although some standards have been determined for cancer therapy, various approaches and treatments are applied for specific to each type of cancer. Biological therapies, such as radiotherapy, chemotherapy, surgery, immunotherapy, hormone therapy, targeted therapies and gene therapy can be used alone or in combination in cancer therapy. However, these methods, known as the gold standard, have advantages as well as disadvantages. Despite the discovery of many chemotherapeutic that inhibit uncontrolled cell division process for the treatment of different types of cancer, serious side effects of these drugs on hematopoietic system, bone marrow and gastrointestinal epithelial cells and hair follicles are a crucial disadvantage. In addition, multi-drug resistance is another important problem in anticancer treatment. Due to problems such as cytotoxicity and drug resistance in existing chemotherapeutic agents, many investigations are being conducted to discover and develop effective anticancer drugs. Previous studies showed that many compounds obtained from natural resources can be used as preventive and therapeutic agents in cancer therapy. These compounds have been shown to increase the effectiveness and tolerance of chemotherapeutic agents when used in combination with chemotherapy or alone in various types of cancers. In recent years, researchers have focused on the anticancer activity of coumarin and coumarin-derived natural compounds among the large number of photochemical, due to their high biological activity and low toxicity. Coumarin is commonly used especially in the treatment of prostate cancer, renal cell carcinoma and leukaemia, and they also have the ability to counteract the side effects caused by radiotherapy. Mahler et al. revealed that combination of coumarin and troxerutin have a positive effect on the treatment of malignity of head and neck radiotherapy. Both coumarin and coumarin derivatives are promising compounds as potential inhibitors of cell proliferation in various carcinoma cell lines.

Roles of coumarin as anticancer agent

Studies conducted on the anticancer activity of coumarin and its derivatives revealed that the mechanism of action of these compounds is generally caspase dependent apoptosis.CYP 2A6, an isoforms of cytochrome P450, metabolizes coumarin to 7-hydroxycoumarin, which has an antiproliferative effect by reducing Bcl expression in various organs and tissues. Bcl-2, a 26 kDa membrane protein, blocks free oxygen radicals, inhibits mitochondrial CYP and suppresses activation of caspase-9, which extends the cell life cycle cumulatively. Thus, it causes carcinogenesis and leads to accumulation of oncogenes mutations in the normal cell. Caspase-9 is activated by Bax, a membrane protein. Over-expression of Bax causes mitochondrial cytochrome C to be released into the cytoplasm through modulation in the mitochondrial membrane. Cytochrome C in the cytoplasm activates caspase-9 and activation of caspase-9 leads to the caspase-3, 6 and 7 activation which break down key cytoplasmic and nuclear proteins.

Coumarin regulate the fate of normal cells by modulating signal transduction pathways containing GTP-binding proteins and reducing Bcl-2 expression. The Bcl-2 protein family consists of Group, which are apoptotic, and Group, which are pro-apoptotic. BH1, BH2 and BH3 in the Bcl-2 protein family and their dimerization determine the sensitivity of a cell to negative stimuli. As the ERK/MAPK pathway actively participates in cell proliferation and cytokine production, this pathway is used as an important target for the development of new anticancer agents.

The general structure activity relationship (SAR) and anti-cancer activity of coumarin are presented in Figure:

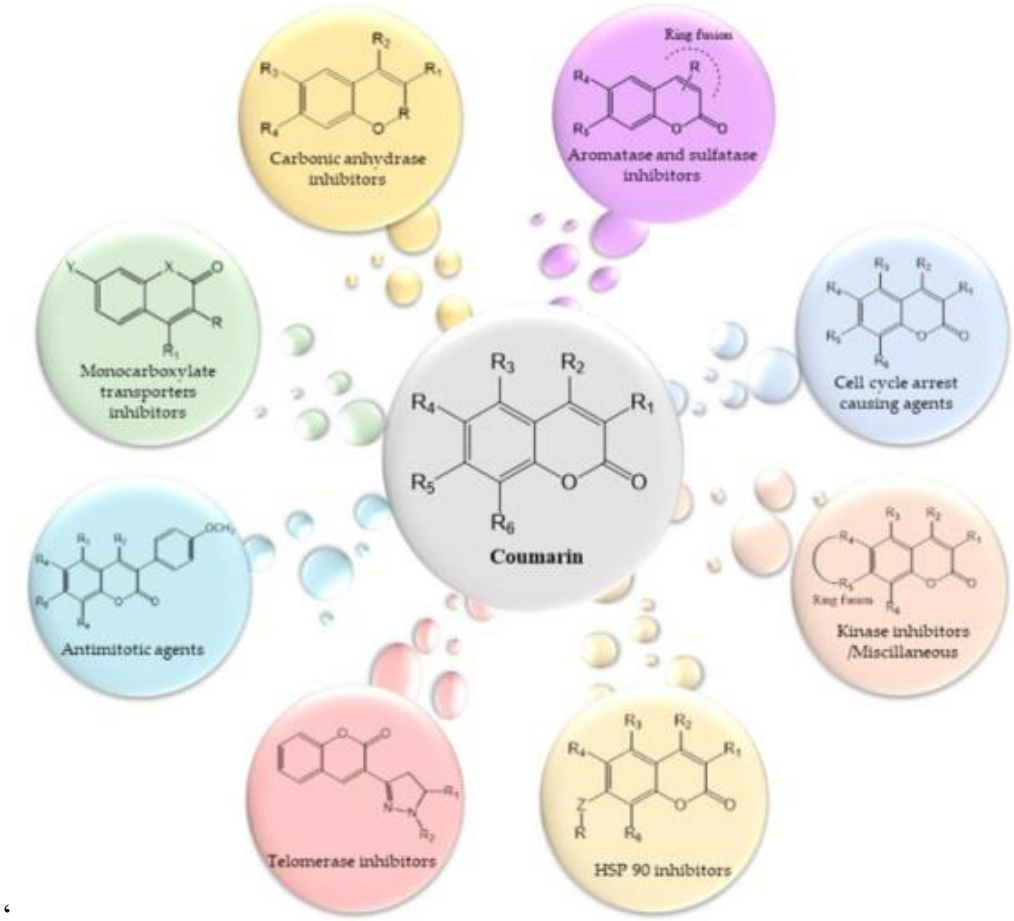


Figure 4: General structure activity relationship (SAR) and anti-cancer activity of coumarin

Roles of coumarin in anticancer activity with structure activity relationship.

A coumarin compound, esculetin, exhibits many pharmacological effects related with cell proliferation and so antitumor efficacy. Polylactide-co-glycoside (PLGA) nano-micelles formulation of esculetin, nano-esculetin, was prepared to treat against insulinoma INS-1 cells which release more insulin than normal beta cells. Results of this study revealed that administration of nano-esculetin decreased the cell viability more significantly than free esculetin in vitro. Free esculetin also decreased the viability of cells in vitro; however, it has been known that nano-formulations exhibit their superior efficacy at in vivo conditions due to

enhanced permeability and retention (EPR) effect. Therefore, nano-formulation of esculetin is thought to be more effective than free esculetin at in vivo conditions.

Coumarin in Breast Cancer

The breast tissue is composed of lobules formed by the glands that produce milk, ducts that allow milk to be discharged and fat and connective tissues. Lobes are formed by combination of the lobules, and each breast has 15–20 lobes. The lobules are connected to each other by milk ducts and milk ducts join towards the nipple. The development and physiological functions of the breast are regulated by hormones. The main hormones that provide the development of breast tissue are estrogens and progesterone.

Breast cancer is a systemic disease that occurs when the cells lining the mammary glands and milk ducts proliferate abnormally, spread to various tissues and organs and continue to grow there. It is a complex disease that affects women physically, psychologically and socially, and ranks first among cancer types seen in women in the world, and also second most common cause of death due to cancer following the lung cancer. In epidemiological studies, the prevalence was found to be 22–26% and the risk of breast cancer-related mortality was around 18%. Risk factors related to breast cancer development are summarized in Table 1.

Table 1: Risk Factors in the Development of Breast Cancer.

Demographic Features	**Gender, Age, Race/Ethnicity**
Reproductive story	The age of menarche, number of birth, first full-term pregnancy age, menopause age, lactation, infertility, miscarriage
Familial/genetic factors	Family history, known or suspected BRCA1/2, p53, PTEN, or other gene mutations related to breast cancer risk
Environmental factors	Radiotherapy to the thorax before 30 years of age, hormone replacement therapy, alcohol use, socioeconomic level, etc.
Other factors	Personal history of breast cancer, number of breast biopsies, atypical hyperplasia or lobular carcinoma in situ, dense breast structure, body mass index (BMI)

Since the breast consists of two main structures, there are two types of breast cancer: lobular cancer developing from the milk secreting part and ductal cancer developing from the milk ducts. The most common type of breast cancer is ductal cancer and accounts for 75% of all breast cancers. Breast cancers are histological divided into two main groups, in situ and invasive carcinomas. In situ carcinoma, malign epithelial cells are limited in ducts and acinus surrounded by basement membrane while in invasive (infiltrative) carcinoma, neoplastic cells cross the basement membrane and show invasion to the stroma. While the malignant breast tumors have been classified traditionally according to their histological appearance, today some subtypes have been defined according to their molecular features. The subtypes of breast cancers have been identified according to the presence of estrogens receptor (ER) in the light of gene expression studies by Perou et al. for the first time. According to this valid classification, ER positive tumors contain gene expression similar to luminal cells of the mammary glands, cytokeratin

profile and markers associated with other luminal cells. In contrast, some of the ER negative tumors are immunohistochemically positive for human epidermal growth factor receptor-2, cerb-B2 (HER2) or HER2 gene amplification may be demonstrated in these tumour cells. This group is known as HER2 positive tumors. HER2 negative luminal non-group tumors show gene expression and immune reactivity similar to normal basal cells of mammary glands. Since ER and progesterone receptor (PR) are also negative in this type of tumors, this group is called basal-like or triple negative tumour group. As a result of studies and meta-analyzes, it was determined that 75% of breast tumors ER and/or PR positive, that is, most tumors are in the luminal group. However, tumors in the luminal group are divided into subtypes as luminal A and B because of their different behaviours. Tumors of luminal a group, which has the highest prevalence among breast cancers, consist of HER2 negative tumors with low proliferative activity, mitotic rate and histological grade. The prognosis of patients with luminal A tumour is good and metastases are often limited to bones. Luminal B tumors are more malignant and the most important difference of this group is that tumors have high proliferation rate. The limit value between luminal A and B is generally considered to be nuclear Ki67 expression immunohistochemically less than 14% of tumour cells. In addition, approximately 30% of HER2 positive tumors are immunohistochemically in the luminal B phenotype.

Despite the development of early diagnosis strategies and advances in treatments, breast cancer is still an important reason of mortality and morbidity. Prognostic factors known in breast cancer are lymph node involvement, tumour size, distant metastasis status, tumour cellular differentiation degree, patient's age, state of hormone receptors in tumour, HER2 over expression, tumour proliferation index, lymph vascular invasion, tumour histology, response to neoadjuvant chemotherapy and hormonotherapy and p53 mutation.

In premenopausal women, high levels of androstenedione compete with aromatase inhibitors in the enzyme complex in cases which estrogens synthesis cannot be completely blocked, and an initial lowering of the estrogens level causes an increase in the level of Gonadotropin. The main source of estrogens in postmenopausal women is the conversion of androstenedione released from the adrenal gland into estrogens through the aromatase enzyme in the peripheral tissues. The aromatase inhibitors (AI) used at this stage lower the plasma estrogens level by inactivating or inhibiting the aromatase. The presence of hormone-induced tumors, including stimulation of the estrogens receptor, has been reported in about one-third of postmenopausal breast cancer patients. In recent preclinical and clinical studies, the synthesis of estrogens receptor agonists/antagonists has gained importance in the prevention and treatment of breast cancer.ER antagonists are commonly used in the treatment of postmenopausal women and hormone-induced breast tumors. Aromatase and sulfatase pathways play a role in the synthesis of estrogens formed only in peripheral tissues. The aromatase pathway ensures that the androgen precursor androstenedione, which is mainly secreted by the adrenal cortex, is converted into estrogens by the aromatase (AR) enzyme complex, while the estrone sulfatase pathway (E1-STS) provides the conversion of the aromatase-induced estrone to estrone sulphate (E1S) by sulfotransferase enzymes. In breast tumors, the activity of the sulfatase enzyme is higher and leads to poor prognosis. The E1-STS pathway is considered the main source of estrogens

formation, which causes a fairly strong response in patients with ER + breast tumors. This approach led to the discovery of new coumarin as STS and AR inhibitors.

AI provides lowering in the level of estrogens and thus prevents breast cancer by reducing cell proliferation, which includes the inhibition of the formation of genotoxic metabolites of estrogens. Genotoxic estrogens metabolites are (i) catechol estrogens that covalently bind to DNA and induce mutations that initiate cancer; (ii) 2-hydroxyl-estradiol forming stable DNA insert; and (iii) 4-hydroxy-estradiol, a potential carcinogenic metabolite that causes 8-hydroxylation of guanine bases leading to estrogens induced indirect DNA damage.

Aromatase inhibitors are the standard option in postmenopausal breast cancer. Previous studies have revealed that benzopyranone substrates, such as 4-benzyl-3-(4'-chlorophenyl)-7-methoxy-coumarin, are stronger competitive AI than aminoglutethimide. It has been reported that the specific interaction of this compound with AR shows a greater decrease in binding to the active site of AR and suppressed the proliferation of AR and ER positive MCF-7 breast cancer cells.

There is an over-expressed ER in the breast tumour cell at an early stage of cancer and during hormonal therapy. Many antitumor agents used in treatment have non-selective effect and acute toxicity so use of these agents in the treatment is limited. Conjugation of cytotoxic drug components to a carrier with selective activity to tumour tissues is an effective strategy in the development of effective antitumor drugs with a high therapeutic index. Studies have shown that combining the cytotoxic agent with steroid hormones provides target selectivity of the conjugate and allow conjugates to accumulate in ER-rich cells as a result of improving antitumor activity and binding to ER.

In a study investigating the antiproliferative efficacy of new bio conjugates containing 3-substituted coumarin and estradiol, highly antiproliferative activity of compounds on non-invasive and invasive breast cancer cell lines (MDA-MB-231/ATCC and NCI/ADR-RES MDA-MB-435) has been revealed.

Cui *et al.* showed the anticancer effects of three synthesized coumarin derived from triphenylethylene (TCHs), occurring through the inhibition of angiogenesis on breast cancer cell lines. Compound TCH-5c inhibited proliferation, resulted in cell death, increased p21 protein expression to induce G0/G1 arrest and changed endothelial cell cytoskeleton organization and migration in EA.hy926 endothelial cells. In addition, this compound inhibited breast cancer cell line derived VEGF secretion, decreased breast cancer cell-induced endothelial cell tube formation in vitro and suppressed SK-BR-3 breast cancer cell-initiated tumour formation in vivo. These results have potential implications in developing new approaches against breast cancer.

Coumarin in Leukaemia

Leukaemia is a clonal disease that results from neoplastic exchange of hematopoietic precursor cells in the bone marrow. Marrow damage occurs when a large number of immature and malignant cells replace normal marrow cells. Thus, decrease begins in the number of platelets involved in blood coagulation and the number of leukocytes involved in defence. This causes intense injuries and bleeding in patients with leukaemia, as well as easy infection. Moreover, the defence mechanism weakens and may cause anaemia and shortness of breath in

advanced stages. Leukaemia has symptoms such as weakness and fatigue, fever, some neurological symptoms, bloating and bleeding in the gums.

Leukaemia is a type of cancer which affects the blood production system (lymphatic system and bone marrow) in the body. Leukaemia is classified as acute or chronic (they are subdivided according to their appearance under the microscope) and according to the spread and development characteristics of the tumour. Generally, acute leukaemia occurs in children, while chronic leukaemia tends to be more common in adults. There are different types of blood cancers according to the cell type (such as myeloid and lymphoid) and the duration of the disease. Some types of blood cancers show a faster and poor prognosis. Leukaemia is more common in childhood than other types of cancers, and 30–35% of cancers in this period are composed of leukaemia. Frequency is 3–4 in 100,000 in children under 15 years of age in western countries.

Although the causes are not known exactly, both genetic and environmental factors are thought to play an important role in leukaemia. Mutations in DNA in somatic cells cause activation of oncogenes or inactivation of tumour suppressor genes. Thus, regulation of cell death and division is damaged. Apart from genetic reasons, this damage is thought to be caused by petrochemicals, radiation, carcinogens and some viruses (e.g., HIV). The purpose of leukaemia treatment is to reduce the number of white blood cells. Since surgery cannot be performed for this purpose, the basis of treatment is the use of cancer drugs, the combination of chemotherapy and radiotherapy, and bone marrow transplantation in cases where chemotherapy is insufficient.

Egan *et al.* have reported that 8-nitro-7-hydroxycoumarin showed cytotoxic properties and induced apoptosis in the tested HL-60 and K562 human leukaemia cell lines. In previous studies investigating the effects of coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and esculetin on the growth, metabolism and cell signal of human tumour cell lines, it was determined that esculetin has the strongest antiproliferative effect on the tested carcinoma cell lines. In a study by Cooke and O ’Kennedy, it has been reported that 7-hydroxycoumarin and esculetin inhibit tyrosine Phosphorylation in EGF-stimulated tumour cells in a time and dose-dependent manner, and the effect can be achieved by reducing tyrosine kinase activity of the EGF receptor.

Esculetin was found to inhibit cell growth and cell cycle progression by inducing G1 phase retention in human leukaemia HL-60 cells in the study by Wang et al. In this study, it has been shown that esculetin provides a significant increase in the level of hypophosphorylated retinoblastoma protein (pRb) and a decrease in CDK4 level, and also significantly up regulation of p27 and down regulation of cycling D1. The results suggested that, as a result of inhibited pRb Phosphorylation, esculetin can inhibit the growth of human leukaemia HL-60 cells by stopping the G1 phase cell cycle. In the study conducted by Jimenez-Orozco et al., 7-hydroxycoumarin was found to have more cytostatic activity than coumarin on the human adenocarcinomas cell line A427, which has pRB positive and homozygous deletions in the p16INK4a gene. However, it has been reported that inhibition of cell cycle in G1/S transition is consistent with the cytostatic effect of 7-hydroxycoumarin and does not create an alteration in cycling D1 mRNA level after transcription.

Taniguchi *et al.,* reported that methoxyinophyllum P, calocoumarin B, and calophyllolide isolated from the leaves of *Rhizophora mucronata* showed cytotoxic effects against HL-60 cells (promyelocytic leukaemia cells) with IC50 of 12.9, 2.6, and 2.2 µM. Furthermore, armenin, hemiterpene ether coumarin, isolated from *Artemisia armeniaca* Lam, showed cytotoxic effects with IC50 equal to 22.5 and 71.1 µM for K562 and HL-60 cells (chronic myelogenous leukaemia cells), respectively.

Coumarin in Malignant Melanoma

Although malignant melanoma (MM) is the third most common skin cancer, it is the skin cancer with the increasing incidence and highest mortality in the world. It is anticipated that 1 out of every 75 people born in 2000 will develop malignant melanoma in any period of their lives, and 20% of them will die due to common disease within 5 years after diagnosis. MM accounts for 1.5% of all cancers and approximately 1% of cancer-related deaths. 40% develop from a previously existing nevus. Intermittent and intense exposure to sunlight is the biggest risk factor among environmental risk factors, especially when combined with endogenous factors (Fitzpatrick 1 and 2 skin types, immune deficiency syndromes and genetic predisposition) .The risk of malignant melanoma increases 1000 times in patients with genetic anomalies such as xeroderma pigment sum (XP). Risk factors related to malignant melanoma development are summarized in Table 2

Table 2: Risk factors in the development of malignant melanoma

Physical conditions	Light skin colour, blond or red hair, light eyes, freckles, or skin burns easily in the sun Sunburn history (formation of one or more severe, vesicle-forming sunburn) Having many or unusual moles
Familial/genetic factors	Family history of melanoma People diagnosed with melanoma in close relatives such as parents, children or siblings
Environmental factors	Life close to the equator or at high altitude Exposure to UV rays from the sun or solarium
Weakened immune system	People with weakened immunity (e.g., those with organ transplants)

The purpose of the treatment of patients with malignant melanoma is to locally control the disease and to prevent lymph node and remote organ spread, if possible. Early diagnosis and adequate primary surgical resection form the basis of malignant melanoma treatment.

Warfarin, which is a coumarin derivative, may play an important role in the treatment of malignant melanoma due to its effects such as preventing tumour spread, stimulating granulocyte, lymphocyte and macrophages. In the study conducted by Thornes *et al.,* coumarin and warfarin have been reported to prevent recurrence of malignant melanoma as macrophage and dose dependent. Velasco-Velazquez et al. revealed that 4-hydroxycoumarin disrupts the actin cytoskeleton in marine melanoma cell line B16-F10 but does not affect benign fibroblastic

cell line B82 and may be useful as adjuvant therapy in melanoma as it prevents the adhesion of tumour cells to extracellular matrix during metastasis.

Coumarin in Renal Cell Carcinoma

Renal cell carcinoma (RCC) is responsible for approximately 3% of tumors seen in adulthood. In recent years, especially with the widespread use of imaging methods, there is an increase in the incidence of RCC at every stage and mortality rates resulting from disease. One third of patients with RCC are metastatic at the time of diagnosis, or one third of them develop metastases despite treatment. Most of the tumors in RCC are associated with large, locally advanced and often lymph node, renal vein or vena cava invasion, and histological, approximately 90% are transparent cell types. Metastasis occurs in many organs, especially lung, liver, bone, adrenals, pancreas, brain, thyroid gland, skin and urethra in order of frequency. While the transparent cell type metastasizes to the lungs more often, papillary type makes it to the lymph nodes and the chromophobes to the liver. The average survival in these patients is 10–12 months, and the 2-year survival rate is 18–20%.

There have been promising developments in prognosis with the introduction of new molecules that make up immunotherapy and targeted therapy, a better understanding of the timing and effectiveness of cytoreductive nephrectomy, and the use of these methods together. Response rates do not exceed 10–15% in immunotherapy using IL-2 and interferon alfa. Tyrosine kinase inhibitors (bevacizumab, sunitinib, sorafenib) used in targeted therapy act by vascular epithelial growth factor (VEGF) receptor blockade and mTOR inhibitors (temsirolimus and everolimus) by reducing regulatory factors that can be induced by hypoxia. These drugs are applied as first and second line treatment in metastatic renal cell carcinoma, resulting in significant improvements in total and disease-free survival times.

It has been reported in clinical studies on metastatic renal cell carcinoma patients that 14 of 45 RCC patients showed positive results with almost no toxic/side effects in case of use of coumarin at the oral dose of 100 mg/day and with the addition of Cimetidine at the dose of 4 × 300 mgs/day from the 15th day of administration. In dose and toxicity studies conducted by Marshall *et al.*, it was determined that coumarin was well tolerated at the applied doses (600–5000 mgs) and it was thought that nausea, which was determined as a common side effect, was caused by the intense aroma of coumarin. Finn et al. reported that 6-nitro-7-hydroxycoumarin and 7,8-dihydroxycoumarin showed irreversible cytotoxic effects in human renal carcinoma cells and non-carcinoma proximal tubular cells. However, it was determined that mentioned compounds were not mutagenic in the Ames test. A derivative, consisting of 1,2,4-triazolin-3-one attached to 4-methylcoumarin, was found to have hopeful activity against RCC cell line. A recent derivative, coufin, a novel indolylcoumarin, displayed potent anticancer activity both in 2D (monolayer culture) and 3D (tumour spheroid culture) by inhibiting microtubule formation and blocking the cell cycle at G2/M. The results obtained suggest that the investigated coumarin may play a potential therapeutic role in the treatment of renal cell carcinoma.

Coumarin in Prostate Cancer

Prostate cancer is the most common solid tissue cancer that occurs with the uncontrolled growth of cells in the prostate gland. Cancer cells primarily grow uncontrolled and spread into the prostate. These then reach the capsule surrounding the prostate, pierce the capsule and

spreads out of the prostate. Unlike benign prostate gland enlargement, prostate cancer does not originate from the centre of the prostate, but from its decentralized, distal centre. Therefore, urinary complaints in prostate cancer disturb the patient in the later period. It is characterized by a very slow growth rate and broad biological variability in terms of hormonal sensitivity. It can spread to nearby organs, the lymphatic system and other parts of the body through the bloodstream during the period of growth and spread. Prostate cancer has a slow course, but the tumour may show a rather aggressive character and spread to the bones and other organs. According to the data of American Cancer Society, men have been reported to have 16.7% risk of development of prostate cancer life-long, and a 2.5% risk of life loss. One in every 5–6 men is at risk of developing prostate cancer throughout their life. When prostate cancer is diagnosed at an early stage, it is among the cancer types with high treatment success. While the treatment options of prostate cancer vary according to the growth rate and spread of cancer and general health status of the patient, the treatment options are surgery, chemotherapy, radiotherapy, hormonal therapy or their different combinations.

In phase I trial studies of 40 patients with metastatic, hormone-naive or hormone-refractory prostate cancer, positive results were obtained with 3 g of coumarin daily in patients with low tumour burden. However, during the 7 years following the study, it was found that prostate-specific antigen (PSA) levels remained stable, with only three bone metastases. In the study of Myers *et al.,* coumarin was found that after 5 days of treatment at concentrations of 0, 10, 50, 100, 250, 500 μg/mL, it inhibits the proliferation of two malignant prostate cell lines (DU145 and LNCaP).

Coumarin and Other Cancers

Taniguchi *et al.,* reported that methoxyinophyllum P, calocoumarin B, and calophyllolide isolated from the leaves of *Rhizophora mucronata* displayed anticancer activity against HeLa cells (cervical cancer) with IC50 equal to 3.8, 29.9, and 36.4 μM.

Clausarin isolated from the root bark of *Clausena harmandiana,* showed high cytotoxic activity which was superior to that of Cisplatin used as a positive control, against hepatocellular carcinoma (HepG2, IC50 = 17.6 ± 2.1 μM), colorectal carcinoma (HCT116, IC50 = 44.9 ± 1.4 μM) and lung adenocarcinomas (SK-LU-1, IC50 = 6.9 ± 1.6 μM) cell lines. From a mechanistic point of view, apoptosis induction was reported as an anticancer mechanism of coumarin.

On the other hand, several studies have been carried out on the synthesis of coumarin to produce coumarin derivatives with improved anticancer effects. Among them, synthetic scopolamine derivatives showed the greatest effects (IC50 < 2 μM) in MCF-7 and MDA-MB 231 cells (human breast adenocarcinomas cell line) as well as in HT29 cells (human colorectal adenocarcinomas cell line). The relationship between the increase in Log P value and the increase in cytotoxic activity was established in this study. Cell cycle arrest was also suggested as the anticancer mechanism of synthetic scopolamine derivatives.

Zhang *et al.* synthesized novel anticancer analogs of geiparvarin using a Bioisosterism transformation method. In their study, it was also shown that adding electron-withdrawing substituents to the benzene rings, such as 7-((1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methoxy)-4H-chromen-4-one, increased their cytotoxic effects in a human hepatoma cell line (QGY-7701,

IC50 = 14.37 ± 9.93) and a colon carcinoma cell line (SW480, IC50 = 11.18 ± 2.16) compared with geiparvarin (IC50 = 17.68 ± 0.40 and 20.34 ± 0.75, respectively).

In addition to the anticancer activity, coumarin has various pharmacological properties, including anti-HIV, antihypertensive, analgesic, Antihyperlipidemic, anti-inflammatory and anticoagulant.

Conclusion:

Because of inadequate capabilities for cancer prevention, diagnosis, and effective treatment, the rate of cancer-related mortality is at an alarming level on a global scale. Coumarin, a privileged scaffold, has an exceptional anticancer profile due to its widespread presence in numerous naturally occurring chemicals. Along with number of mechanisms discussed in the chapter the photodynamic effect of coumarin has attracted more interest.

Several bacteria, plants, fungi, and ascidians produce coumarins, which are common secondary metabolites. Many of these substances from different natural sources have been researched for their CA inhibitory properties and potential biomedical applications, primarily as anticancer agents targeting hypoxic tumours also. The potent coumarin derivatives have demonstrated incredible capacity to control the prospective anticancer actions on the basis of various substitution patterns.

References:

1. J. Immunotoxicity of Epicutaneously Applied Anti-Coagulant Rodenticide Warfarin. Intercept Ltd.; Hampshire, UK: 1999. Pp. 245–263.
2. Bruneton J. Pharmacognosy, Phytochemistry, Medicinal Plants. 2 nd ed. Intercept Ltd.;Hampshire, UK: 1999. Pp. 263–277.
3. Ojala T. Ph.D. Thesis. University of Helsinki; Helsinki, Finland: 2001. Biological Screening of Plant Coumarins.
4. Lacy A., O'Kennedy R. Studies on coumarins and coumarin-related compounds to determine their therapeutic role in the treatment of cancer. Curr. Pharm. Des. 2004;10:3797–3811.
5. Vogel, A. (1820). "Darstellung von Benzoesäure aus der Tonka-Bohne und aus den Meliloten- oder Steinklee-Blumen" [Preparation of benzoic acid from tonka beans and from the flowers of melilot or sweet clover]. Annalen der Physik (in German). 64 (2):161–166.
6. Vogel, A. (1820). "De l'existence de l'acide benzoïque dans la fève de tonka et dans les fleurs de mélilot" [On the existence of benzoic acid in the tonka bean and in the flowers of melilot]. Journal de Pharmacie (in French). 6: 305–309.
7. Guibourt, N. J. B. G. (1820). Histoire Abrégée des Drogues Simples [Abridged History of Simple Drugs] (in French). Vol. 2. Paris: L. Colas. Pp. 160–161.
8. Guibourt, N. J. B. G. (1869). Histoire Naturelle des Drogues Simples (6 th ed.). Paris: J. B.Baillière et fils. P. 377.
9. Guillemette, A. (1835). "Recherches sur la matière cristalline du mélilot" [Research into the crystalline material of melilot]. Journal de Pharmacie. 21: 172–178.
10. Perkin, W. H. (1868). "On the artificial production of coumarin and formation of its homologues". Journal of the Chemical Society. 21: 53–63. Doi:10.1039/js8682100053.
11. Yang SM, Shim GY, Kim BG, Ahn JH. 2015. Biological synthesis of coumarins in

Escherichia coli. Microb Cell Fact 14:65.

12. Zhu JJ, Jiang JG. 2018. Pharmacological and nutritional effects of natural coumarins and their structure-activity relationships. Mol Nutr Food Res 62:1701073.
13. Ananthakrishnan, R.; Chandra, Preeti; Kumar, Brijesh; Rameshkumar, K. B. 2018. "Quantification of coumarin and related phenolics in cinnamon samples from south India using UHPLC-ESI-QqQLIT-MS/MS method". International Journal of Food Properties. 21: 50–57.
14. Cassia Cinnamon as a Source of Coumarin in Cinnamon-Flavored Food and Food Supplements in the United States J. Agric. Food Chem., 61 (18), 4470–4476
15. Leal, L. K. A. M.; Ferreira, A. A. G.; Bezerra, G. A.; Matos, F. J. A.; Viana, G. S. B. 2000. "Antinociceptive, anti-inflammatory and bronchodilator activities of Brazilian medicinal plants containing coumarin: a comparative study". Journal of Ethnopharmacology. 70 (2): 151–159.
16. Lino, C. S.; Taveira, M. L.; Viana, G. S. B.; Matos, F. J. A. (1997). "Analgesic and antiinflammatory activities of Justicia pectoralis Jacq. And its main constituents: coumarin and umbelliferone". Phytotherapy Research. 11 (3): 211–215.
17. Ieri, Francesca; Pinelli, Patrizia; Romani, Annalisa (2012). "Simultaneous determination of anthocyanins, coumarins and phenolic acids in fruits, kernels and liqueur of Prunus mahaleb L". Food Chemistry. 135 (4): 2157–2162. Doi:10.1016/j.foodchem.2012.07.083.hdl:2158/775163. PMID 22980784
18. Egan D, O'Kennedy R, Moran E, Cox D, Prosser E, Thornes RD. Drug Metab. Rev. 1990;22:503.
19. Borges F, Roleira F, Milhazes N, Santana L, Uriarte E. Curr. Med. Chem. 2005;12:887.
20. Murray RDH, Mendez J, Brown SA. The Natural Coumarins: Occurrence, Chemistry and Biochemistry. New York: Wiley; 1982.
21. Lacy A, O'Kennedy R. Curr. Pharm. Des. 2004;10:3797.
22. Gottlieb OR, Herrmann K, Murray RDH, Ohloff G, Pattenden G. Progress in the Chemistry of Organic Natural Products. New York: Springer-Verlag; 1978.
23. Amaral, R.; dos Santos, S.; Andrade, L.; Severino, P.; Carvalho, A. Natural Products asTreatment against Cancer: A Historical and Current Vision. Clin. Oncol. 2019, 4, 1562.
24. Devji, T.; Reddy, 26.C.; Woo, C.; Awale, S.; Kadota, S.; Carrico-Moniz, D. Pancreatic anticancer activity of a novel geranylgeranylated coumarin derivative Bioorganic Med. Chem. Lett. 2011, 21, 5770–5773.
25. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2019. CA. Cancer J. Clin. 2019, 69, 7–34.

GENOPROTECTIVE EFFECT OF D-PINITOL ISOLATED FROM AERIAL PARTS OF SOYBEAN PLANTS AGAINST DOXORUBICIN-INDUCED GENOTOXICITY EVALUATED BY IN VITRO COMET ASSAY IN VERO CELL LINES

Murugesan Sudha

Department of Pharmacology,

Faculty of Pharmacy, Dr. M.G.R. Educational and Research Institute,

Deemed to be University, Chennai, Tamil Nadu, India.

Corresponding author E-mail: kaviyasudha7@gmail.com; sudha.pharm@drmgrdu.ac.in

Abstract:

Doxorubicin is a chemotherapeutic agent with a genotoxic effect on normal cells at its therapeutic dose itself. D-Pinitol is an abundantly available carbohydrate in Soybean plants and has been proven for antioxidant and anti-inflammatory activities. Our investigation was examined by *in vitro* comet assay to explore the genoprotective effect of D-Pinitol in normal cells against Doxorubicin-induced genotoxicity in Vero cell lines. *In vitro* comet assay treatment groups were Vero Cell lines with culture medium served (control group), Doxorubicin (0.15µg/ml), D-Pinitol (0.05×10^3 mM, 0.125×10^3 mM, and 0.25×10^3 mM) alone, and pretreatment with D-Pinitol (0.05×10^3 mM, 0.125×10^3 mM, and 0.25×10^3 mM) before Doxorubicin (0.15µg/ml) treatment. When compared to the control group, D-Pinitol alone treated groups showed no significant changes in the percentage of DNA damage. For the evaluation of the genoprotective effect of D-Pinitol, the % DNA damage in the D-Pinitol, and Doxorubicin simultaneously treated groups were compared to the Doxorubicin alone treated group. The results showed that Doxorubicin-induced genotoxic effect in Vero cell lines was significantly reduced by D-Pinitol in a dose-dependent manner by reducing DNA damage. Our findings confirmed that D-Pinitol had no genotoxic effect, and it showed a genoprotective effect against Doxorubicin-induced genotoxicity.

Keywords: Genotoxicity; Doxorubicin; D-Pinitol; *in vitro* Comet assay.

Introduction:

Doxorubicin (DOX) is a potent antibiotic of anthracycline class in anticancer drugs. Although DOX is very useful for treating various types of human cancers, having severe side effects at its therapeutic dose is one of the most undesired outcomes of using it. Hence management of its side effects is essential to achieve patient's treatment, tolerability, and overall quality of life. Remesh, (2012). Previous reports suggested that oxidative stress and free radicals' production are the main reasons for DOX-induced cardiotoxicity, cytotoxicity, and genotoxicity. Hajra *et al.,* (2018). Apart from these side effects, DOX also induces severe inflammatory responses in various organs, including the kidney, liver, blood vessels, and intestine. The previous reports also showed an increase in pro-inflammatory cytokine levels after DOX administration. Dong *et al.,* (2016); Xu *et al.,* (2008). Since DOX has an important role in cancer treatment, it is very crucial to minimize its toxic effects on normal cells. This reduction in DOX-induced toxicity to normal cells can be attained by simultaneous administration of free radical scavenging agents, antioxidants and anti-inflammatory agents. Decreasing DOX's toxicity by attenuating oxidative stress and pro-inflammatory mediators is a forthcoming therapeutic approach against DOX-induced toxicity. Hajra *et al.,* (2018).

Hence, the compounds that can scavenge free radicals and reduce the levels of pro-inflammatory mediators will safeguard the normal cells from DNA damage when they subjected to the genotoxic agents. Čabarkapa, *et al.,* (2014). D-Pinitol has been identified as a major carbohydrate present in *Glycine max* L. Merr. Streeter, (2001); Sripathi and Poongothai *et al.,* 2013). It has been reported for its free radical scavenging capacity (Orthen *et al.,* (1994)), antioxidant activity (Balasubramanian *et al.,* (2014); Sivakumar *et al.,* (2010)), anti-inflammatory activity (Kim J.C. *et al.,* (2005); Singh R.K. *et al.,* (2001)), hepatoprotective effect (Choi *et al.,* (2009); Song, *et al.,* (2008)), and Cardioprotective effect (Kim J.-I. *et al.,* (2005)). These therapeutic effects of D-Pinitol may be useful in the prevention of the genotoxic effect of DOX on normal cells. This research's main goal was to evaluate the genoprotective effect of D-Pinitol in the Vero cell line from the DNA damage induced by DOX through *in vitro* comet assay.

The *in vitro* Comet assay presents a visual technique to analyze DNA damage in the cells when treated with chemicals inducing toxic effects. Visvardis *et al.,* (2000). Hence this assay is an advantageous, fast, and effective *in vitro* method for studying the genotoxicity of chemical agents. Tice *et al.,* (2000); Anderson *et al.,* (1994).

Materials and Methods:

Materials Required:

Doxorubicin HCL (TCI chemicals, India), D-Pinitol isolated from aerial parts of Soybean (*Glycine max* L. Merr.,) plants, DMEM medium (Gibco, USA), Fetal Bovine Serum (Gibco, USA), antibiotic solution (Gibco, USA), Ethidium Bromide (Merck, India), low melting agarose (Merck, India), Normal agarose (Merck, India), Phosphate Buffer Saline Solution (Himedia, India) and Olympus BX 50 microscope (Olympus Optical Co., Germany).

Methodology - *In vitro* Comet Assay:

Cell line and cell culture:

Vero cell lines (African green monkey kidney cells) were cultured in liquid Dulbecco's Modified Eagle's medium (DMEM) enriched with 10 percentage Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5 percentage CO_2 stored at 37ºC.

Table 1: Treatment protocol

Groups	Labeled	Treatment
I	Control	Vero Cell lines with culture medium
II	Positive Control	Doxorubicin 0.15µg/ml
III	Tests	D-Pinitol 0.05×10^3 mM
IV		D-Pinitol 0.125×10^3 mM
V		D-Pinitol 0.25×10^3 mM
VI		Doxorubicin 0.15µg/ml + D-Pinitol 0.05×10^3 mM
VII		Doxorubicin 0.15µg/ml + D-Pinitol 0.125×10^3 mM
VIII		Doxorubicin 0.15µg/ml + D-Pinitol 0.25×10^3 mM

Selection of doses of Doxorubicin and D-Pinitol were based on Abd-ElAziz *et al.*, (2014) and Al-Ashaal *et al.,* (2012), respectively.

Briefly, Vero cells were incubated at 37°C for 24 h in a humidified 5% CO_2 incubator after they were seeded (density of 10,000 cells/well) in a six-well plate. After the wells were washed with sterile Phosphate Buffer Saline Solution (PBS), the cell lines were treated with D-Pinitol and DOX as per the treatment protocol given in Table.1. Trypsinization was done before harvesting of cells in a 1.5 ml tube. The microscopic slides were coated first with 200 µl of 0.75 % normal melting agarose (the first layer) and 100 µl of 0.5% low melting agarose (the second layer). Next, the slides were distributed with 20 µl cell suspensions in 60 µl of 0.5% low melting agarose (the third layer). Then incubation of slides was done in cell lysis buffer (2.5 M NaCl, 0.2 M NaOH, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% dimethyl sulfoxide, pH =10.0) for overnight at 4°C. After that, the slides were immersed in double-distilled water three times, followed by 20 min incubation with unwinding solution (3M NaOH). The slides were subsequently placed in a horizontal gel electrophoresis tank containing electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH =13). The electrophoresis was conducted at 25 V (1 V/cm, 300 mA) for 25 min. The incubation of slides was done in neutralization buffer (0.4 M Tris–HCl, pH = 7.5) for 10 min followed by immersion in ultrapure water for three times and then air-dried. The staining of cells was done with 50 µl of ethidium bromide (5 mg/L) and then the cells were observed under a fluorescent microscope. 15 minutes after staining, the comets of each group were examined on the microscope at 100 X magnification. In order to minimize extra DNA damage, all steps in the procedure were carried out in dim light. The percentage of DNA damage events was calculated by manual counting. Comets were visually scored and classified into five classes (Fig.4.) based on the level of DNA migration. Anderson *et al.,* (1994). The experiment was performed in triplicate for each group and, analysis was performed on 100 cells for each experiment. Singh N.P., *et al.,* (1988); Nandhakumar *et al.,* (2011).

Statistical analysis:

Statistical analysis was performed by One way ANOVA method. Values are expressed as mean, and for n = 6. All data were analyzed with the GraphPad Prism 8.0.1 software. A difference at $P < 0.05$ was considered statistically significant.

Results and Discussion:

We assessed the effect of D-Pinitol for its ability to prevent DOX-induced genotoxicity. The values were represented as the mean number of different classes of comets that occurred in all groups (Table.2 and Fig.1) and as the percentage of DNA damage (Table.3 and Fig. 2). Images of comets that occurred in all groups were shown in Fig.3. The obtained data elucidates that all three doses of D-Pinitol did not express any significant differences in the percentage of DNA damage values in D-Pinitol alone treated groups when compared to the control group. While increased levels of DNA damage was detected in cells treated only with DOX (0.15µg/ml), a significant reduction in the levels of DNA damage was seen in the Vero cell lines treated simultaneously with DOX (0.15µg/ml) and three different concentrations of D-Pinitol (0.05×10^3 mM, 0.125×10^3 mM and 0.25×10^3 mM) when compared with DOX alone treated group (Tables.2 and Table.3 & Fig.1 and Fig. 2).

Table 2: Number of different classes of comet events that occurred in Vero cell lines

Nature of Comet events	Group I (Vero Cell lines and culture medium)	Group II (DOX 0.15µg/ml)	Group III (D-P 0.05× 10^3 mM)	Group IV (D-P 0.125× 10^3 mM)	Group V (D-P 0.25× 10^3 mM)	Group VI (DOX 0.15µg/ml + D-P 0.05× 10^3 mM)	Group VII (DOX 0.15µg/ml + D-P 0.125× 10^3 mM)	Group VIII (DOX 0.15µg/ml + D-P 0.25× 10^3 mM)
Class 1	94.33 ± 1.453	29.67 ± 4.372	94.67 ± 0.882	94 ± 0.577	93.33 ± 0.667	41.33 ± 3.283	53.33 ± 3.528	81 ± 2.082
Class 2	5.667 ± 1.453	33 ± 0.577	5.333 ± 0.882	6 ± 0.577	6.667 ± 0.667	25.33 ± 1.764	21 ± 1.155	5 ± 0.577
Class 3	0	7.333 ± 0.667	0	0	0	15 ± 1.528	13.33 ± 0.882	11.33 ± 0.882
Class 4	0	18.67 ± 2.333	0	0	0	15.33 ± 0.882	10.33 ± 1.202	2 ± 0.577
Class 5	0	11.33 ± 1.764	0	0	0	3 ± 0.577	2 ± 0.577	0.667 ± 0.333

Abbreviation: DOX – Doxorubicin; D-P – D-Pinitol

Table 3: Effect of D-Pinitol on Doxorubicin-induced genotoxicity by *in vitro* comet assay in Vero cell line

Criterion	Group I (Vero Cell lines and culture medium)	Group II (DOX 0.15µg/ml)	Group III (D-P 0.05× 10^3 mM)	Group IV (D-P 0.125× 10^3 mM)	Group V (D-P 0.25× 10^3 mM)	Group VI (DOX 0.15µg/ml + D-P 0.05× 10^3 mM)	Group VII (DOX 0.15µg/ml + D-P 0.125× 10^3 mM)	Group VIII (DOX 0.15µg/ml + D-P 0.25× 10^3 mM)
% DNA Damage	5.667 ± 1.453	70.33 ± 4.372 ^{a*}	5.333 ± 0.882 aNS	6 ± 0.577 aNS	6.667 ± 0.667 aNS	58.67 ± 3.283 a*bNS	46.67 ± 3.528 a*b#	19 ± 2.082 a@b*

Abbreviation: DOX – Doxorubicin; D-P – D-Pinitol. Mean ± SEM, n=6, where, a- Group II, III, IV, V, VI, VII & VIII compared with Group I. b- Group VI, VII & VIII compared with Group II. * $P < 0.001$, $^{\#}$ $P < 0.01$ & $^{@}$ $P < 0.05$.

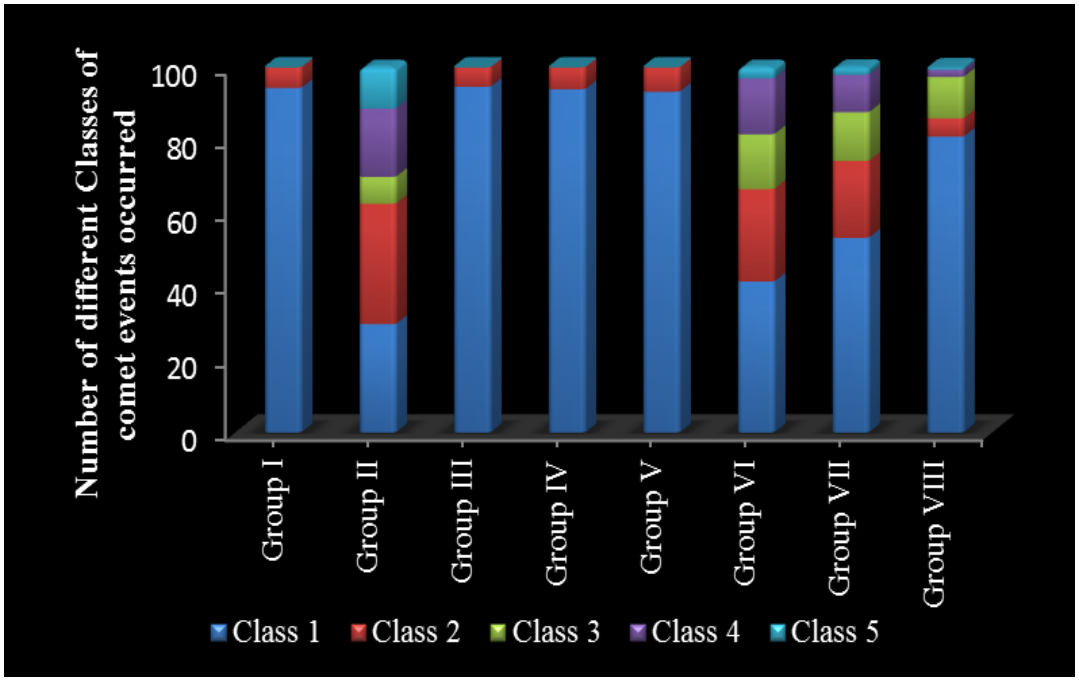


Figure 1: Histogram of number of different Classes of Comet events that occurred in Vero cell line

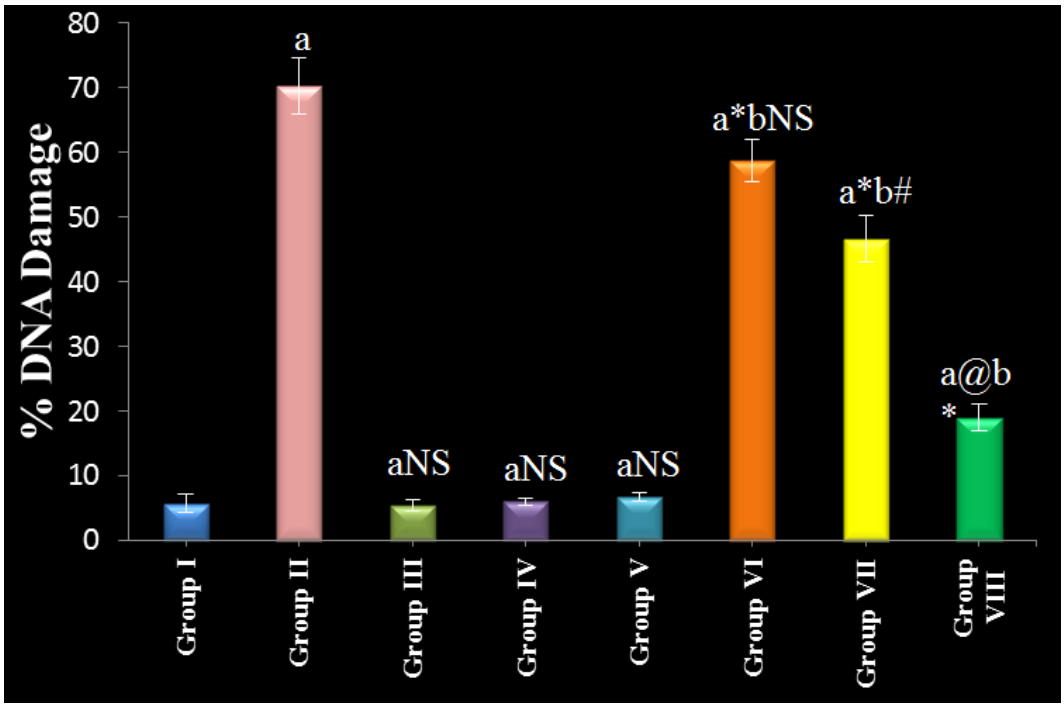


Figure 2: Histogram of genoprotective effect of D-Pinitol on Doxorubicin-induced genotoxicity by *in vitro* comet assay in Vero cell line

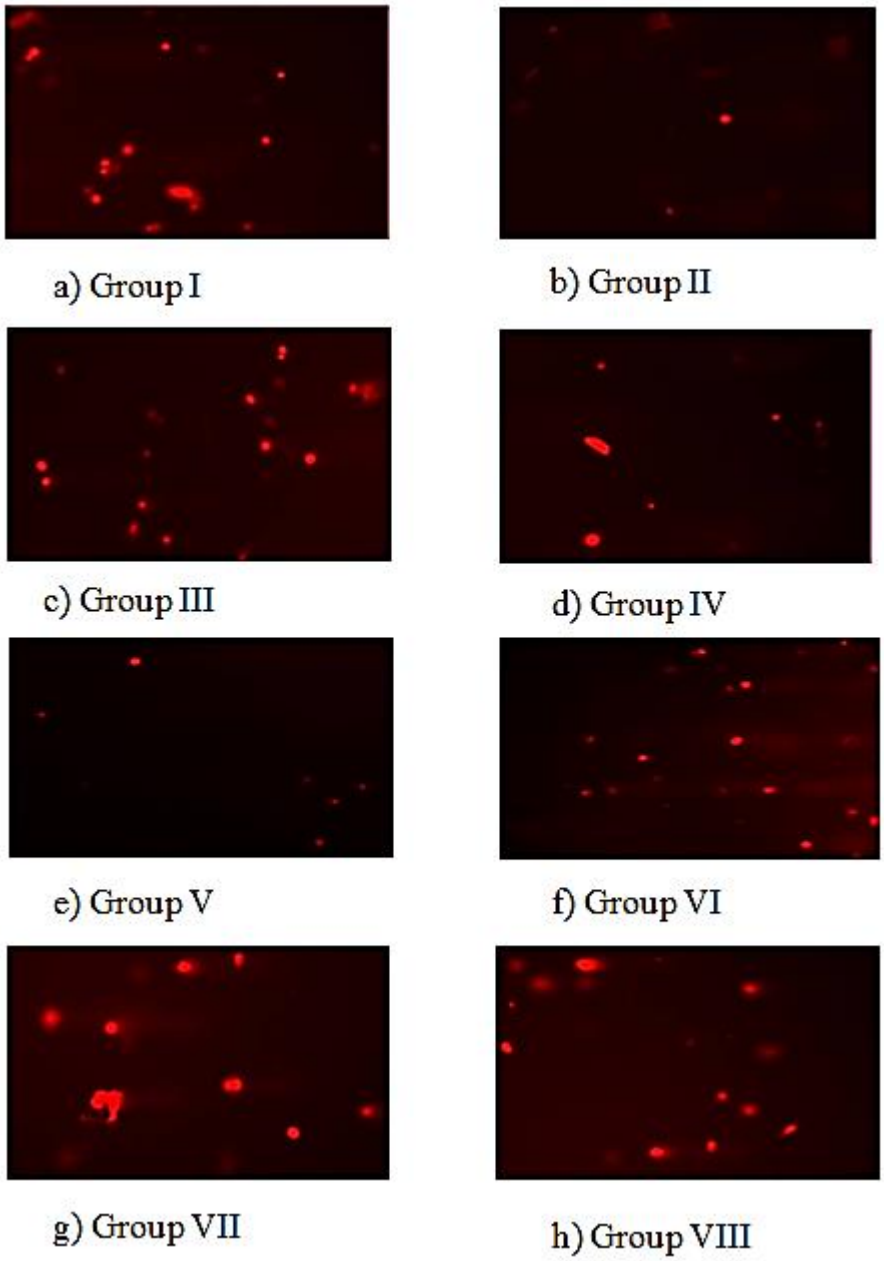


Figure 3: Images of Comets visualized by comet assay in Vero cell lines

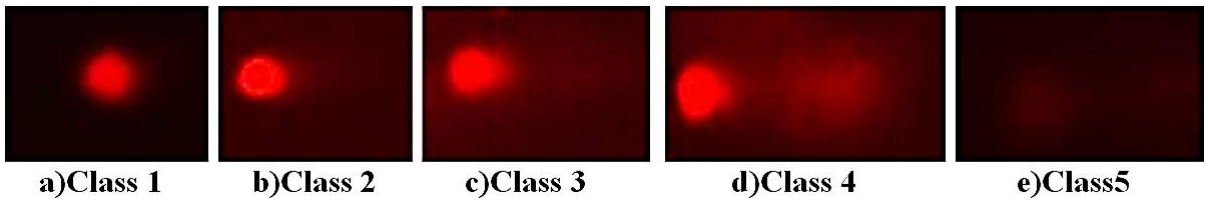


Figure 4: Images of Classes of Comet events: a) Class 1 - no damage, <5%; b) Class 2 - low level damage, 5–20%; c) Class 3 - medium level damage, 20–40%; d) Class 4 - high level damage, 40–95%; e) Class 5 - total damage, >95%.

All three doses of D-Pinitol hopefully decreased the induction of DNA damage by DOX. Concentration-response of D-Pinitol indicated that protection against DOX - induced DNA damage was more intense with an increase in D-Pinitol concentration. The increased DNA

damage reduction was observed in the Vero cell lines pretreated with 0.25×10^3 mM concentration of D-Pinitol. Hence the higher concentration of D- Pinitol showed the more significant protective effect.

The examination of dietary components with antioxidant and anti-inflammatory activities has gotten much consideration because the essential factors in the generation and progression of numerous chronic diseases were oxidative stress and inflammation. Arts and Hollman *et al.,* (2005). D-Pinitol has been shown its beneficial effects in several experimental models of diseases emerging and worsening because of oxidative stress and inflammation. Its antioxidant (Balasubramanian *et al.,* (2014); Sivakumar *et al.,* (2010)) and anti-inflammatory properties (Singh R.K., *et al.,* (2001); Kim J.C. *et al.,* (2005)) were well reported.

DOX, an anthracycline anticancer agent, can induce reactive oxygen species (ROS) generation with severe inflammatory responses. DNA damage caused by inflammatory Reactive Oxygen Species can inevitably lead to disturbance of genetic stability. Hence the production of free radicals and pro-inflammatory mediators is the primary mechanism responsible for DOX genetic toxicity and DNA damage to normal cells. Quiles *et al.,* (2002).

The genotoxic evaluation of D-Pinitol by *in vitro* comet assay in Vero cell lines indicated that this compound did not induce any genotoxic effects. This investigation also showed the capacity of D-Pinitol to protect oxidative DNA damage caused by DOX in Vero cell lines. In our results, all concentrations of D-Pinitol showed genoprotective effect against DOX - induced genotoxicity. The protective effect of D-Pinitol is possibly due to the result of expression of several molecular pathways such as ROS scavenging effect through its antioxidant capacity and attenuation of pro-inflammatory mediators by its anti-inflammatory property. Genoprotective effect of D-Pinitol was more pronounced at the larger concentration than smaller concentrations.

Conclusion:

Our findings showed that D-Pinitol was not genotoxic to Vero cell lines. Our investigation confirmed the genoprotective effect of D-Pinitol against DOX - induced genotoxicity by protecting normal cell lines from DNA damage.

Acknowledgments:

Authors thank the authorities of Adhiparasakthi College of Pharmacy for providing necessary facilities.

References:

1. Abd-ElAziz, M.A., Al-Shdefat, R.I., Al-Saikhan, F.I., (2014): Genoprotective and Genotoxic Effects of Thymoquinone on Doxorubicin-Induced Damage in Isolated Human Leukocytes, Tropical Journal of Pharmaceutical Research, 13(12), 2015-2020.
2. Al-Ashaal, H.A., Fahmy, M.A., Melek, F.R., Aly, N.H., Hasan, Z.M., (2012): Effect of supplemented soybean (*Glycine max* L) diet and extracts on aluminum sulfate-induced genotoxicity, Toxicological & Environmental Chemistry, 94(5): 965-986.
3. Anderson, D., Yu, T-W., Phillips, B.J., Schmezer, P., (1994): The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 307(1), 261–271.

4. Arts, I.C.W., Hollman, P.C.H., (2005): Polyphenols and disease risk in epidemiologic studies, The American Journal of Clinical Nutrition, 81(1), 317–325.
5. Balasubramanian, M.P., Rengarajan, T., Rajendran, P., Nandakumar, N., Nishigaki, I., Haribabu, L., (2014): Free radical scavenging and antioxidant activity of D-pinitol against 7, 12- Dimethylbenz(a) Anthracene induced breast cancer in Sprague Dawley rats. Asian Pacific Journal of Tropical Disease, 4(5), 384-390.
6. Čabarkapa, A., Živković, L., Žukovec, D., Djelić, N., Bajić, V., Dekanski, D., Spremo-Potparević, B., (2014): Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using *in vitro* comet assay with human peripheral leukocytes, Toxicology in Vitro, 28(3), 451–456.
7. Choi, M.-S., Lee, M.-K., Jung, U.J., Kim, H.-J., Do, G.-M., Park, Y.B., Jeon, S.-M., 2009: Metabolic response of soy pinitol on lipid-lowering, antioxidant and hepatoprotective action in hamsters fed-high fat and high cholesterol diet, Molecular Nutrition & Food Research, 53(8), 751 – 759.
8. Dong, L., Wang, L., Chen, Q., Qi, H., Wang, C., Wang, C, Zhang, J., (2016): Doxorubicin-Induced Systemic Inflammation Is Driven by Upregulation of Toll-Like Receptor TLR4 and Endotoxin Leakage, Cancer Research, 76(22), 6631- 6642.
9. Hajra, S., Patra, A.R., Basu, A., Bhattacharya, S., (2018), Prevention of doxorubicin (DOX)-induced genotoxicity and cardiotoxicity: Effect of plant derived small molecule indole-3-carbinol (I3C) on oxidative stress and inflammation, Biomedicine & Pharmacotherapy, 101, 228–243.
10. Kim, J.C., Shin, J.Y., Shin, D.H., Kim, S.H., Park., S.H., Park, R.D., Park, S.C., Kim, Y.B., Shin, Y.C., (2005): Synergistic Antiinflammatory Effects of Pinitol and Glucosamine in Rats, Phytotherapy Research, 19, 1048–1051.
11. Kim, J.-I., Kim. J.C., Kang, M.-J., Lee, M.-S., Kim, J.-J. Cha, I.-J., (2005): Effects of Pinitol isolated from soybeans on glycaemic control and cardiovascular risk factors in Korean patients with type II diabetes mellitus: a randomized controlled study, European Journal of Clinical Nutrition, 59, 456–458.
12. Nandhakumar, S., Parasuraman, S., Shanmugam, M.M., Rao, K. R., Chand, P., Bhat, B.V., (2011): Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay), Journal of Pharmacology and Pharmacotherapeutics, 2(2), 107-111.
13. Orthen, B., Popp, M., Smirnoff, N., (1994): Hydroxyl radical scavenging properties of cyclitols, Proceedings of the Royal Society of Edinburgh, Section B. Biological Sciences, 102, 269-272.
14. Quiles, J.L., Huertas, J.R., Battino, M., Mataix, J., Ramirez-Tortosa, M.C. (2002): Antioxidant nutrients and adriamycin toxicity, Toxicology, 180(1), 79-95.
15. Remesh, A., (2012): Toxicities of anticancer drugs and its management, International Journal of Basic & Clinical Pharmacology, 1(1), 2-12.
16. Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L. (1988): A simple technique for quantitation of low levels of DNA damage in individual cells, Experimental Cell Research, 175, 184–191.

17. Singh, R.K., Pandey, B.L., Tripathi, M., Pandey, V.B. (2001): Anti-inflammatory effect of (+)-pinitol, Fitoterapia, 72, 168-170.
18. Sivakumar, S., Palsamy, P., Subramanian, S.P. (2010): Attenuation of oxidative stress and alteration of hepatic tissue ultrastructure by D-pinitol in streptozotocin-induced diabetic rats, Free Radical Research, 44(6), 668–678.
19. Song, Y.-S., Zhou, Y., Park, C.-M., Cho, C.-W., (2008): Protective Effect of Pinitol against D-Galactosamine-Induced Hepatotoxicity in Rats Fed on a High-Fat Diet, Bioscience, Biotechnology, and Biochemistry, 72 (7), 1657–1666.
20. Sripathi, S.K., Poongothai, G., (2013): A review on insulinomimetic Pinitol from plants, International Journal of Pharma and Bio Sciences, 42, 992 – 1009.
21. Streeter, J.G., (2001): Simple partial purification of D-Pinitol from soybean leaves, Crop Science, 41, 1985–1987.
22. Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., and Sasaki, Y.F., (2000): Single cell gel/Comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing, Environmental and Molecular Mutagenesis, 35, 206-221.
23. Visvardis, E.-E., Haveles, K.S., Pataryas, T.A., Margaritis, L.H., Sophianopoulou, V., Sideris, E.G., (2000): Diversity of peripheral blood mononuclear cells as revealed by a novel multiple microgel "Comet assay", Environmental and Molecular Mutagenesis, 36, 32-39.
24. Xu, Z., Lin, S., Wu, W., Tan, H., Wang, Z., Cheng, C., Lu, L., Zhang, X., (2008): Ghrelin prevents Doxorubicin-induced cardiotoxicity through TNF-alpha/NF-κB pathways and mitochondrial protective mechanisms, Toxicology, 247 (2-3), 133–138.

AN APPRAISAL ON FOOD-FOOD INTERACTIONS

Syeda Nishat Fathima*[1] and S. M. Firdous[2]
[1]Department of Pharmacology,
Jayamukhi College of Pharmacy, Narsampet, Warangal-506032, Telangana, India
[2]Department of Pharmacology,
Calcutta Institute of Pharmaceutical Technology & Allied Health Sciences,
Howrah, West Bengal-711316, India
*Corresponding author E-mail: syeda.nishu.fathima@gmail.com

Abstract:

Food-food interactions referred to as the dynamic and complex relationships between different food components when consumed together. Understanding these interactions is crucial for optimizing nutrient absorption, bioavailability, metabolism and overall health outcomes. This article provides a concise overview of the various aspects and implications of food-food interactions.

Keywords: Food-food interactions; Inhibition; Synergism; Health outcomes

Introduction:

The effect of food on a person may be different than expected because that food interacts with another nutrient the person is taking, food, beverages, dietary supplements the person is consuming. A drug interaction is a situation in which a substance affects the activity of a drug, i.e., the effects are increased or decreased, or they produce a new effect that neither produces on its own. These interactions may occur out of accidental misuse or due to lack of knowledge about the active ingredients involved in the relevant substances. These interactions, known as food-food interactions, include a complex interplay of nutritional, physiological, chemical, and sensory factors. Understanding the dynamics of these interactions is vital for optimizing nutrient digestion, absorption, bioavailability, metabolism and overall health outcomes. [1-2]

Types of interactions:

The different types of food interactions that can be encountered are:

1. Complementary interactions
2. Conflicting interactions
3. Enhancing interactions
4. Inhibitory interactions
5. Synergistic interactions

Complementary interactions:

When two or more meals are combined, they can improve the nutritional value of the meal by offering a balance of necessary nutrients or complementary ingredients. This form of food-food interaction is known as a complementary interaction. By supplying a balance of necessary nutrients or complimentary ingredients, complementary interactions can improve the nutritional content of the meal. Optimising nutrient intake and general health can be accomplished by consuming a variety of nutrient-rich meals and mixing them in various ways. [3]

Following are a few instances of complimentary interactions in food:

1. Legumes and Grains: Consuming grains like rice, quinoa, and barley along with legumes like beans, lentils, and chickpeas can offer a balanced intake of protein and important amino acids. While grains are high in methionine, legumes are high in lysine. Together, these meals can offer a full amount of protein and boost nutritional intake.
2. Spinach and Lemon: Consuming spinach along with lemon can improve the body's ability to absorb iron. Iron is abundant in spinach, but it is present in a form that the body cannot readily absorb. Iron's bioavailability can be increased by converting it into a more soluble state with the aid of the vitamin C in lemons.
3. Avocado with tomato: Lycopene, a carotenoid with antioxidant characteristics present in tomatoes, can be better absorbed when consumed with avocado. Lycopene's bioavailability and absorption can both be enhanced by avocado's beneficial lipids.
4. Salad with Nuts and Seeds: Eating a salad with nuts and seeds can give you a good mix of protein, healthy fats, and minerals. Healthy fats, protein, fibre, and micronutrients like vitamin E, magnesium, and zinc are all abundant in nuts and seeds. A meal that is nutrient-dense and balanced can be made by eating them along with a variety of vegetables and leafy greens.
5. Muesli with Berries: Including berries in your muesli can help you get the right amount of complex carbs, fibre, and antioxidants. Berries are a wonderful source of antioxidants like anthocyanins, while muesli is a good supply of complex carbs and fibre. Together, these can make for a well-rounded and nutrient-rich dinner.

Conflicting interactions:

Some foods can conflict with each other and alter the body's ability to utilize a particular food or drug, or cause serious side effects. contradictory interactions may prevent some nutrients or substances from being absorbed, used, or metabolically processed, which may result in nutrient shortages or other adverse health consequences. For nutrients to maximise their absorption and overall wellness, it is of the utmost importance to be conscious of these interactions and consume nutrient-rich foods as a component of a balanced and varied diet. For example, consuming milk and citrus fruits together can cause curdling and upset stomach due to the acidity in the fruits. [4] The following are a few examples of Conflicting food interactions:

1. Calcium and Iron: Consuming calcium-rich foods beside foods which contain iron can prevent the body from absorbing iron. For individuals who have low iron levels, calcium's ability to bind to iron and prevent absorption can be problematic. It is advised to consume foods that are rich in calcium and foods abundant in iron separately to optimise the absorption of both minerals.
2. Coffee and Calcium: Consuming coffee in conjunction with foods high in calcium may hinder the body from properly absorbing the mineral. Caffeine can decrease calcium's absorption and increase calcium excretion in the urine. To maximise calcium absorption, it is advised to reduce coffee consumption and consume calcium-rich foods separately.
3. Copper and zinc: Consuming copper- and zinc-rich foods simultaneously may hinder the body from absorption and utilisation of both of them. High intakes of zinc can prevent copper from being absorbed and cause a deficiency in copper, whereas high intakes of

copper can prevent zinc from being absorbed and cause a deficiency in zinc. To optimise their absorption, it is advised to consume foods that contain copper and zinc in moderation and in a balanced diet.

4. Alcohol and Nutrient Absorption: Drinking alcohol can prevent certain vitamins and minerals, including thiamine, folate, and vitamin B12, from being absorbed and utilised by the body. Alcohol can increase these nutrients' excretion in the urine and reduce their absorption in the small intestine. Nutrient deficits and general health problems might result from chronic alcohol use.
5. Grapefruit with Medications: Consuming grapefruit or grapefruit juice with certain medications can alter their metabolism and elevate blood levels, which may cause unwanted consequences. Medications including statins, calcium channel blockers, and immunosuppressants can be metabolised by enzymes that are inhibited by compounds found in grapefruit. When using drugs, it is advised to either avoid grapefruit or grapefruit juice altogether.

Enhancing interactions:

Enhancing interactions are a form of food-food interaction when the combination of two or more foods results in in an increase in the bioavailability or potency of a specific nutrient or chemical. Enhancing interactions can increase the potency or bioavailability of particular nutrients or chemicals and offer more health advantages. Optimising nutrient absorption and utilisation can be accomplished through consuming a variety of food that contain nutrients and incorporating them in different ways. [5] Here are some essential insights about enhancing interactions:

1. Vitamin D and calcium: Combining calcium- and vitamin D-rich food may enhance both minerals' absorption and absorption. Together, calcium and vitamin D consumption can strengthen bones and reduce the possibility of osteoporosis since vitamin D improves the body's capacity to absorb calcium.
2. Iron with vitamin C: Iron can be more effectively absorbed by the body if utilised together with foods that are rich in vitamin C. Iron that's found in plant-based meals, referred to as non-heme iron, is easier for the body to absorb with the help of vitamin C. Foods that contain vitamin C, such as citrus fruits or bell peppers, ought to be consumed together with foods rich in iron, such as spinach or lentils.
3. Turmeric with Black Pepper: The bioavailability of curcumin, the active ingredient in turmeric, can be enhanced by combining turmeric with black pepper. Piperine, a substance found in black pepper, can enhance curcumin's absorption and enhance its anti-inflammatory and antioxidant benefits.
4. Tomatoes with Olive Oil: Lycopene, a carotenoid with antioxidant characteristics present in tomatoes, might be better absorbed when consumed with olive oil. Healthy fats included in olive oil can enhance lycopene absorption and boost its bioavailability.
5. Green Tea and Lemon: When green tea and lemon are combined, the catechins—a class of chemicals in green tea with antioxidant and anti-inflammatory properties—are more easily absorbed. Vitamin C, which can be found in lemons, can enhance the body's ability to absorb catechins.

Inhibitory interactions:

Inhibitory food-food interactions refer to the interactions that occur between specific foods or food components, resulting in a reduction in the absorption, metabolism, or effectiveness of certain nutrients or bioactive compounds. These interactions can impact the overall nutritional value of a meal and affect the body's ability to utilize and benefit from the ingested nutrients. [6] Here are some important notes on inhibitory interactions:

1. Antinutrients: Certain compounds found in food, such as phytates, oxalates, and tannins, can bind to minerals and inhibit the body from absorbing them. For instance, the phytates in seeds, nuts, and legumes can bind to and impede the absorption of minerals like iron, calcium, and zinc. Antinutrient concentrations can be lowered and mineral absorption improved by cooking, soaking, and sprouting.
2. Protein inhibitors: certain foods include proteins that may hinder other proteins from being effectively metabolised and absorbed, such as the trypsin inhibitors found in soybeans and other legumes. The levels of protein inhibitors can be reduced and protein digestion enhanced through cooking, soaking, and fermenting.
3. Food and drug interactions: Some foods and drugs can interact, reducing the effectiveness or absorption of the former. For instance, dairy products' calcium and magnesium might hinder the absorption of antibiotics like tetracycline and ciprofloxacin.
4. Food intolerances and allergies: Consuming certain foods could lead to intolerances or allergies, which may hinder the body from absorbing and using nutrients. For instance, individuals with lactose intolerance may have difficulties with digestion and have having difficulty absorbing calcium and other nutrients from dairy products.
5. Fibre interactions: Consuming a lot of fibre, particularly that derived from whole grains, could hinder certain nutrients, like iron and zinc, from being absorbed. However, ingesting fibre along with other foods can also delay the breakdown and absorption of carbs and increase feelings of satiety.
6. Nutrient Binding: Certain foods contain compounds that can bind to nutrients to form complexes that are difficult for the body to absorb. For instance, the phytic acid in nuts, legumes, and whole grains can bind to minerals like calcium, iron, and zinc, preventing their absorption. Similar to oxalates, calcium can bind to oxalates in foods like spinach and rhubarb to create insoluble crystals, which inhibit calcium absorption.
7. Enzyme Inhibition: Certain foods consist of natural substances that can hinder some digestive enzymes from activating effectively, inhibiting the absorption and digestion of nutrients. For example, the tannins in tea and coffee can prevent the action of digestive enzymes like amylase and protease, which will alter the manner in which carbohydrates and proteins are digested out.
8. Competitive Absorption: In the gastrointestinal tract, specific nutrients use similar absorption pathways. They may compete for absorption when ingested in significant quantities combined, resulting in lower uptake of one or more nutrients. One such is the rivalry for absorption between zinc and iron. Zinc absorption might be hampered by high dietary iron levels and vice versa.

Synergistic interactions:

Synergistic food-food interactions are those between particular foods or food components that produce increased or amplified health benefits as compared to consuming the individual foods independently. The combined effect of the foods in these interactions is greater than the sum of each food's individual effects. It can be advantageous to promote general health and wellbeing to comprehend and take advantage of synergistic food-food interactions. Synergistic interactions can improve protein quality, increase antioxidant effects, improve flavour and texture, and support glycaemic regulation. [7-8] Here are some significant aspects regarding synergistic interactions:

1. Nutrient Enhancement: Certain dietary combinations can improve the bioavailability and absorption certain nutrients. Consuming foods that contain vitamin C, such as citrus fruits, together with foods abundant in plant-based iron, such as spinach, can, for instance, improve the absorption of non-heme iron. Iron is more easily absorbed by the body when it undergoes transformation to a more absorbable form through the presence of vitamin C. The absorption of fat-soluble vitamins, such as vitamin D, can be improved by consuming foods high in healthy fats.
2. Antioxidant Synergy: Certain meals contain substances with antioxidant capabilities that combine to offer increased defence against oxidative stress. For instance, pairing foods high in vitamin E, like nuts and seeds, with foods rich in vitamin C, like berries, might increase the meal's overall antioxidant capacity. Together, vitamins E and C help other nutrients thrive, enhancing their antioxidant properties.
3. Phytochemical Interactions: Phytochemicals, such as polyphenols and carotenoids, are found in many foods and have an array of health benefits. These phytochemicals can interact synergistically when ingested together, boosting their unique benefits. For instance, eating tomatoes with olive oil improves the bioavailability of the carotenoid lycopene, which is more readily absorbed when there is dietary fat present.
4. Protein Complementation: By combining several plant-based sources of protein, a full protein profile that includes all necessary amino acids can be produced. For instance, combining legumes with grains or seeds can result in a more well-balanced amino acid profile, guaranteeing an adequate intake of protein.
5. Glycemic control: Consuming a combination of nutrients that have different glycemic index values can help regulate blood sugar levels and promote satiety. Consuming carbohydrates along with fibre, good fats, or protein, for instance, might delay the digestion and absorption of carbohydrates and provide an enduring source of energy.

Conclusion:

In conclusion, food-food interactions encompass a wide range of effects on nutrient absorption, glycaemic response, bioactive properties, and sensory experiences. Recognizing and harnessing these interactions can contribute to improved dietary choices, nutrient utilization, and overall well-being. Further research is necessary to unravel the intricate mechanisms and implications of food-food interactions, enabling evidence-based recommendations for optimal nutrition and overall well-being.

References:

1. Delwiche, J. F. (2004). The impact of perceptual interactions on perceived flavor. Food Quality and Preference, 15(2), 137–146. https://doi.org/10.1016/s0950-3293(03)00041-7
2. Bushra, R., Aslam, N., & Khan, A. Y. (2011). Food-drug interactions. Oman medical journal, 26(2), 77–83. https://doi.org/10.5001/omj.2011.21
3. D'Alessandro, C., Benedetti, A., Di Paolo, A., Giannese, D., & Cupisti, A. (2022). Interactions between Food and Drugs, and Nutritional Status in Renal Patients: A Narrative Review. Nutrients, 14(1), 212. https://doi.org/10.3390/nu14010212
4. Spanakis, M., Patelarou, E., & Patelarou, A. (2022). Drug-Food Interactions with a Focus on Mediterranean Diet. Applied Sciences, 12(20), 10207. https://doi.org/10.3390/app122010207
5. Rodriguez-Fragoso, L., & Reyes-Esparz, J. (2013). Fruit/Vegetable-Drug Interactions: Effects on Drug Metabolizing Enzymes and Drug Transporters. InTech. doi: 10.5772/48283
6. Galanakis, C. M., & Drago, S. R. (2022). Introduction. In Elsevier eBooks (pp. 1–18). https://doi.org/10.1016/b978-0-323-85052-0.00003-9
7. Jacobs, D. R., Jr, Gross, M. D., & Tapsell, L. C. (2009). Food synergy: an operational concept for understanding nutrition. The American journal of clinical nutrition, 89(5), 1543S–1548S. https://doi.org/10.3945/ajcn.2009.26736B
8. Natarajan, T. D., Ramasamy, J. R., & Palanisamy, K. (2019). Nutraceutical potentials of synergic foods: a systematic review. Journal of Ethnic Foods, 6(1). https://doi.org/10.1186/s42779-019-0033-3

THE PROTECTIVE ROLE OF SPIRULINA ON DOXORUBICIN INDUCED GENOTOXICITY IN GERM CELLS OF RATS

M. Sudha*[1] and S. Kavimani[2]

[1]Department of Pharmacology, Faculty of Pharmacy,
Dr. M.G.R. Educational and Research Institute,
Deemed to be University, Chennai, Tamil Nadu, India.
[2]Department of Pharmacology, College of Pharmacy,
Mother Theresa Postgraduate & Research institute of Health Sciences,
Pondicherry Medical University, Gorimedu, Puducherry - 605006.
* Corresponding author E-mail sudha.pharm@drmgrdu.ac.in, kaviyasudha7@gmail.com, drskavimani@yahoo.co.in

Abstract:

Doxorubicin is a potent antitumor agent used against many cancers. It has the potential for inducing genotoxicity in normal cells including somatic and germ cells. Hence, it is very important to reduce its toxicity to the normal cells. This goal can be achieved by concurrent administration of free radical scavenging agents, like antioxidants. In the present study, adult male albino rats of Wistar strain were administered with Doxorubicin (3 mg/kg body weight, intraperitonially), once in a week for 35 days and Spirulina (250 mg/kg body weight, oral) daily prior to Doxorubicin administration for 35 days. Doxorubicin treated rats showed a significant decrease in sperm count and increase in the sperm head abnormalities. However, the combined treatment of Spirulina with doxorubicin restored the sperm counts and decreases the incidence of sperms with head abnormalities. This study indicated that administration of Spirulina prior to Doxorubicin might prevent the germ cell toxicity in male rats.

Keywords: Doxorubicin, Spirulina, Germ cell toxicity, Genotoxicity, Antioxidants

Introduction:

Genotoxicity refers to the capability of substances to damage DNA and/or cellular components regulating the fidelity of the genome. Eastmond DA *et al.*, (2009). The main problem posed by anticancer drugs is that they target not only the tumour, but also other cells, thus causing the same damage to both abnormal and normal cells. Granados-Principal *et al.*, (2010).

Doxorubicin, an anthracycline antibiotic, is one of the most widely used anticancer drugs. The main anticancer action of Doxorubicin is believed to involve DNA damage through inhibition of topoisomerase II. http://faculty.ksu.edu.sa. Quiles JL, *et al.*, (2002). Doxorubicin causes the generation of free radicals and the induction of oxidative stress, associated with cellular injury. Injac R and Strukelj B, (2008). The free radical generation by Doxorubicin may participate as cardiotoxicity and genotoxicity in normal human cells. http://faculty.ksu.edu.sa;Quiles JL, *et al.*, (2002). It was shown that Doxorubicin induces chromosome aberrations in murine bone marrow cells and in spermatocytes. Chromosome aberrations were sustained in the surviving spermatogonial cells. Doxorubicin also increased the

frequency of meiotic micronuclei in male rats, which showed impaired fertility after being treated. Baumgartner A, *et al.,* (2004).

Genotoxicity of doxorubicin will lead to genetic damage in germ cells. Male germ cells generally much more sensitive to chemical exposure than female germ cells. Decreased weights of the genital organs, an extremely decreased number of sperm, low sperm motility, a low implantation rate and a decreased number of live fetuses were also observed in rats. Infertility is a common side effect of chemotherapy that bears a substantial impairment on the quality of life for young survivors of cancer. http://faculty.ksu.edu.sa.

Spirulina is 50-70% protein by weight and contains a rich source of vitamins especially vitamin B_{12}, β-carotene (provitamin A), vitamin E. It also contains carbohydrates like rhamnose, fructose, ribose, mannose and some minerals like copper, magnesium, zinc, potassium and iron. Beside γ-linolenic acid, it also contains a host of other phytochemicals that have potential health benefits. Spirulina contains phycocyanin (7% dry weight basis) and polysaccharides, both of them have antioxidant properties. Ray S, *et al.,* (2007); Belay A, (2002).

Previous studies have demonstrated that Spirulina exhibits antioxidant property in various oxidative conditions that cause tissue injury. Miranda MS, *et al.* (1998); Premkumar K, *et al.,* (2001). Spirulina treatment reduced the Cyclophosphamide induced testicular spermatogenic cell damage due to genotoxic effect, thereby showing its protective effect on germ cells. Chamorro-Cevallos G, *et al.,* (2008).

The aim of this study was to investigate the genotoxic effect of Doxorubicin on germ cells of male rat and to highlight the protective effect of Spirulina on this genotoxicity.

Materials and Methods:

Animals:

Male albino rats weighing between 170 and 220 g were used for the study. The animals were fed *ad libitum* with standard pellet diet and had free access to water.

Drugs and chemicals used:

Doxorubicin was purchased from V.S. Biotech, Chennai. Spirulina was obtained as gift sample from Antenna Nutritech. All other chemicals used were of analytical grade.

Experimental design:

Animals were randomly selected and divided into four groups with five animals in each group. Doxorubicin (3mg/kg) was reconstituted with normal saline and administered intraperitonially. Spirulina (250mg/kg) was dissolved in normal saline and administered through oral route. Khan M, *et al.,* (2005). The volume of administration to each animal was 10 ml/kg in all the cases. Animals were sacrificed by cervical dislocation 35 days after the last injection, following which the testes and epididymides were isolated.

Dosing schedule:

All the animals were treated for 35 days and the weekly dosing schedule for each group was as follows, Group 1 received normal saline and served as control, Group 2 received Spirulina (250mg/kg) daily, Group 3 received Doxorubicin (3mg/kg) once in a week and served as positive control and Group 4 received Spirulina (250mg/kg) daily followed by Doxorubicin (3mg/kg) on day seven. After 48 hours of the last injection of either doxorubicin or vehicle,

blood was collected for serological analyses. Epididymides were removed, cleared off the adhering tissues.

Epididymal sperm count:

Animals were sacrificed by cervical dislocation. The cauda epididymis was removed and placed in a normal saline. The epididymis was minced into small pieces to allow the sperms to swim out. The sperm suspension thus obtained was centrifuged at 1000 rpm for 5 min. After centrifugation, 1 ml of the supernatant was taken and the epididymal sperm count was determined using Neubauer's hemocytometer. Data were expressed as number of sperms per mg weight of epididymis. Padmanabhan S, *et al.,* (2009).

Sperm Head Abnormality Test:

Animals were sacrificed by cervical dislocation. The cauda epididymis was removed and placed in normal saline solution. The epididymis was minced into small pieces to allow the sperms to swim out. The sperm suspension thus obtained was stained with 2% eosin solution and kept undisturbed for 1 hour. Smears were prepared using the above solution, air dried and fixed with absolute methanol for 5 min. 200 sperms per animal were examined for sperm head morphological abnormalities at 1000X magnification. Sperm head morphology was scored under the category of normal, sperm without hook, amorphous head, banana head and bent at cephalocaudal junction. Data were shown in terms of percentage of abnormal sperms. Padmanabhan S, *et al.,* (2009).

Statistical analysis:

Results of all the above estimations have been indicated in terms of mean ± SEM. Difference between the groups was statistically determined by Student 't' test. The average data generated for the groups of rat treated with Spirulina were compared with our earlier reported respective data on vehicle control group of rat treated with 0.9% NaCl at the rate of 1ml/100 g body weight. The average data generated from the groups of rats treated with Spirulina and Doxorubicin were compared with our earlier reported respective data on rats treated with Doxorubicin 3mg/kg alone. Choudhury RC, *et al.,* (2000). $P < 0.001$ was considered to be statistically significant.

Results:

Epididymal sperm count:

All animals were survived during the experimental period. Significant decrease in the sperm counts was observed in Doxorubicin (3mg/kg) treated group as compared to the control group ($P < 0.01$). There was a significant restoration in the sperm counts in the groups which received Spirulina treatment (250 mg/kg day) prior to Doxorubicin ($P < 0.05$) (Table 1).

Table 1: Effect of Doxorubicin alone and along with Spirulina on epididymal sperm count.

Groups	Sperm Count ($x10^6$/mg epididymis)
Control (0.9% saline)	18±1.2
Spirulina (250 mg/kg)	$18±1.2^{NS}$
Doxorubicin (3mg/kg)	$8±2.3^{**}$
Doxorubicin (3mg/kg) + Spirulina (250 mg/kg)	$14±1.0^{*}$

Values are expressed as mean ± SEM. Doxorubicin was compared with Control. Doxorubicin + Spirulina was compared with Doxorubicin. *P <0.05, **P<0.01, ***P<0.001.

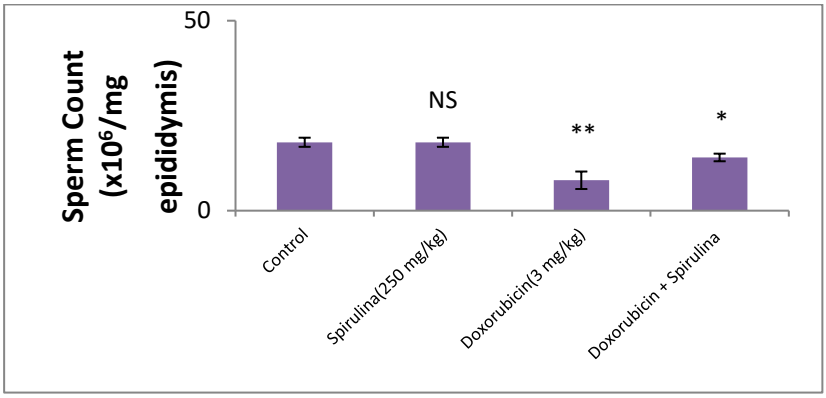


Figure 1: Effect of Spirulina administration on the Epididymal sperm count of Doxorubicin treatment

Sperm Head Abnormality:

Sperm head abnormalities were classified into amorphous head, banana shaped head, head without hook and bent at cephalocaudal junction. The sperm head abnormalities were found to increase significantly in the group which received Doxorubicin (3mg/kg) for 35 days from 53 to 150 as compared to the control groups ($P < 0.001$). A significant decrease in the percentage of abnormal sperms was observed in the group pre-treated with the Spirulina (250mg/kg) from 15% to 7.4% in comparison with the Doxorubicin treated group ($P < 0.001$) (Table 2).

Table 2: Effect of doxorubicin alone and along with Spirulina on Sperm Head Abnormalities.

Groups	Number of sperms examined/ number of animals	Normal sperms	Sperm Head Abnormalities				Total sperm head abnormality	%Abnormal sperm (mean ± SEM)
			Amorphous head	Banana shaped head	Head Without hook	Bent at cephalocaudal junction		
Control	1000/5	947	17	3	18	15	53	5.3±0.9
Spirulina (250 mg/kg)	1000/5	955	17	4	13	11	45	$4.5±1.1^{NS}$
Doxorubicin (3mg/kg)	1000/5	850	41	23	59	27	150	$15±0.7^{***}$
Doxorubicin (3mg/kg) + Spirulina (250 mg/kg)	1000/5	926	24	5	27	18	74	$7.4±0.8^{***}$

Values are expressed as mean ± SEM. Doxorubicin was compared with Control. Doxorubicin + Spirulina was compared with Doxorubicin.*** P <0.001.

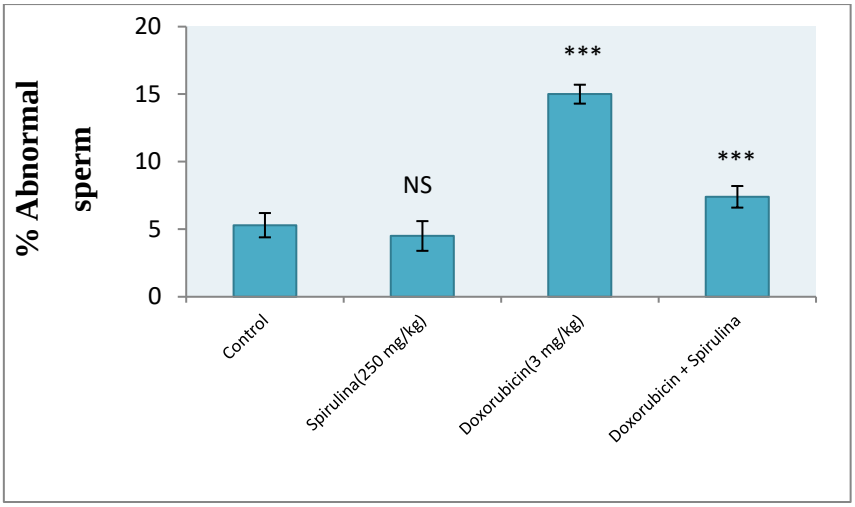


Figure 2: Effect of Spirulina administration on sperm head abnormalities expressed as of abnormal sperm

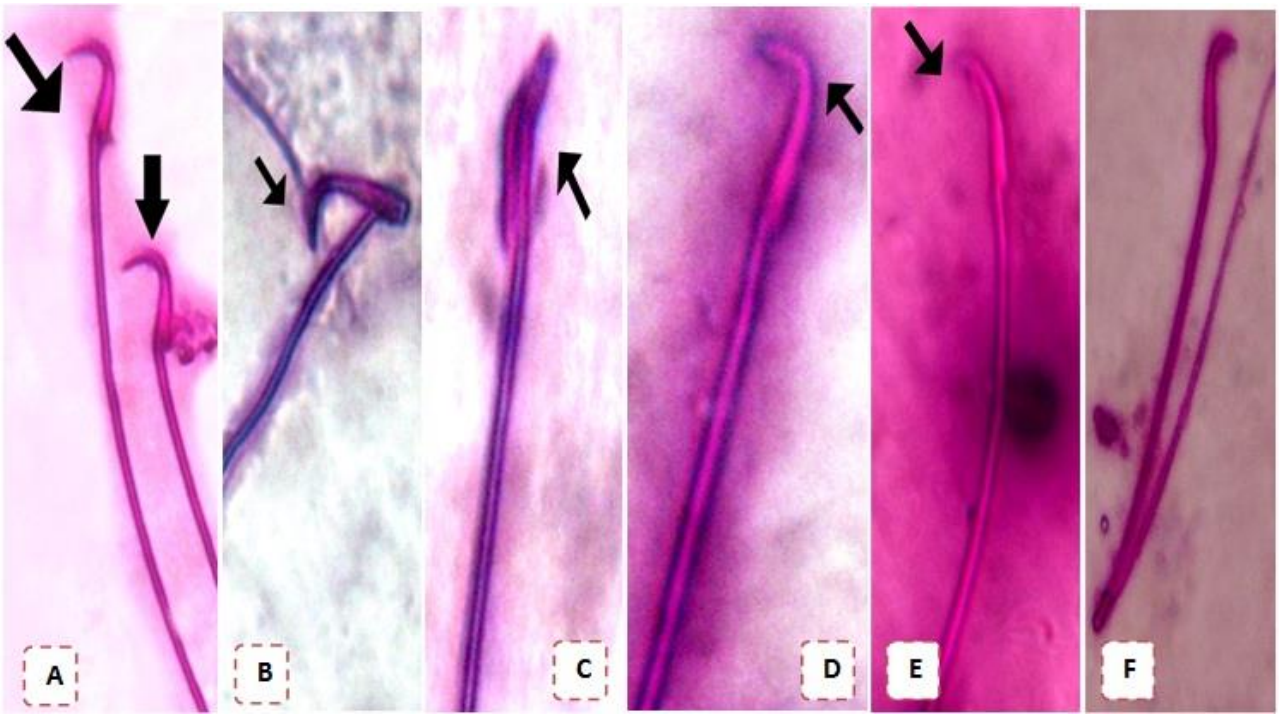


Figure 3: Sperm head abnormalities -(A) Normal sperm; (B) sperm head bent at cephalocaudal junction; (C) amorphous head sperm; (D) sperm with banana shaped head; (E) sperm without hook; (F) sperm with bent tail

Discussion:

Doxorubicin is one of the widely used cytotoxic agents known to disturb spermatogenesis and testicular functions. It has been shown that Doxorubicin-induced cytotoxicity is mainly concentrated in early spermatogenic cells, which undergo rapid proliferation and differentiation. Kang J, *et al.*, (2002). It has been reported that Doxorubicin induced testicular toxicity may be the consequence of oxidative stress. Kalender Y, *et al.*, (2005); Sikka SC, (1996); Sanocka D and Kurpisz M, (2004).

Two different pathways of free radical formation by Doxorubicin have been described. The first implicates the formation of a semiquinone free radical. Granados-Principal S, *et al.*, (2010); Quiles JL, *et al.*, (2002);Suominen JS, *et al.*, (2003). The semiquinone radical can be transformed to a C7 radical that can also mediate cellular damage. The reduction of doxorubicin by two electrons generates a secondary alcohol metabolite, Doxorubicinol. Jahnukainen K, *et al.*, (2000).

In the second pathway, Doxorubicin free radicals come from a non-enzymatic mechanism that involves reactions with iron. For example, Fe^{3+} reacts with Doxorubicin in a redox reaction, after which the iron atom accepts an electron and a Fe^{2+}- Doxorubicin free radical complex is produced. Quiles JL, *et al.*, (2002); Suominen JS, *et al.*, (2003); Jahnukainen K, *et al.*, (2000). This iron-Doxorubicin complex can reduce oxygen to hydrogen peroxide and other active oxygen species. Quiles JL, *et al.*, (2002);Prahalathan C, *et al.*, (2005).

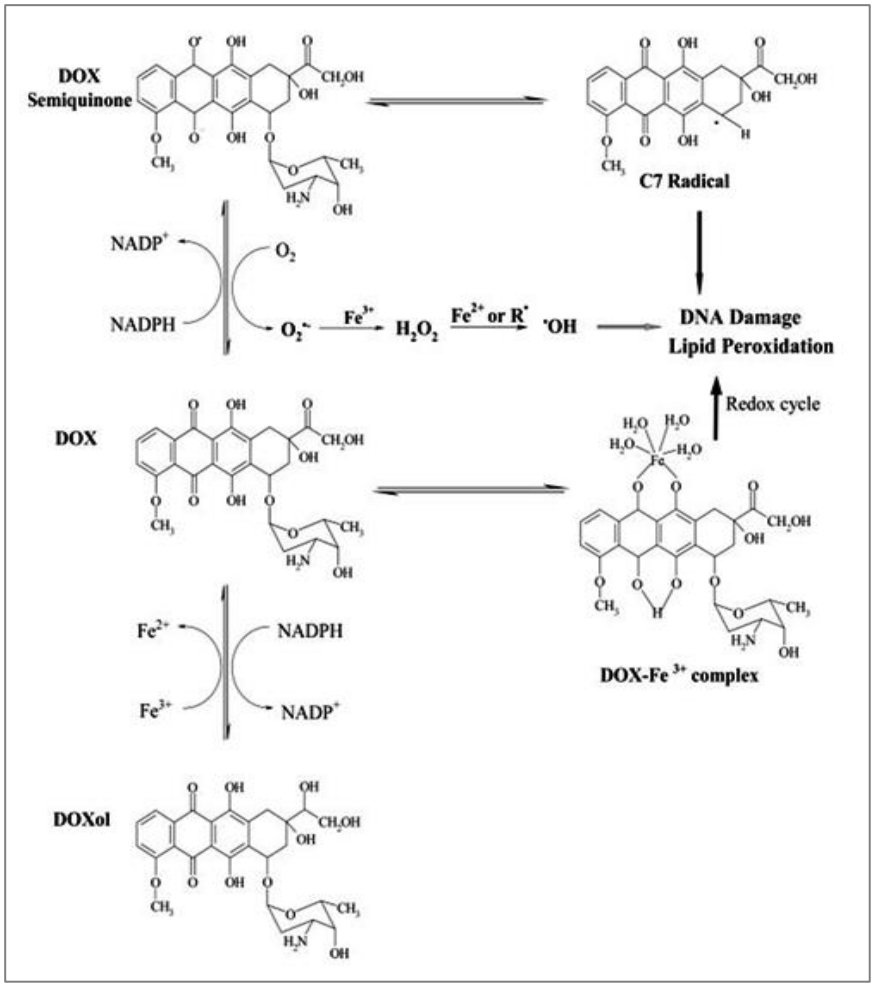


Figure 4: Illutration of doxorubicin-mediated redox cycling. Xu X, *et al.*, (2005)

Increased oxidative damage to sperm membranes, proteins and DNA is associated with lterations in signal transduction mechanisms that can be detrimental to male fertility. Kalender Y, *et al.*, (2005); Sikka SC, (1996); Sanocka D and Kurpisz M, (2004).

Thus, the combination of the drug delivery together with a potent antioxidant may be the appropriate approach to reduce the toxic side effect of Doxorubicin. Prahalathan C, *et al.*, (2006).

Spirulina, blue green microalgae, has been used since ancient times as a source of food because of its high protein and nutritional value. The chemical composition of Spirulina indicates that it has phenolic acid, tocopherol and β-carotene, which are known to exhibit antioxidant properties. Premkumar K, *et al.,* (2004) Previous studies have shown that C-phycocyanin has the ability to chelate metals including free iron. Bhat VB and Madyastha KM, (2001); Romay C, *et al.,* (2003). The cardioprotective effect of Spirulina could also be due to the scavenging of hydroxyl, peroxynitrite and chelating of free iron. Khan M, *et al.,* (2005).

Spirulina treatment reduced the Cyclophosphamide induced testicular spermatogenic cell damage due to genotoxic effect, thereby showing its protective effect on germ cells. Chamorro-Cevallos G, *et al.,* (2008). Spirulina extracts have been shown to inhibit buccal cancers in animal models and buccal squamous cell carcinoma. Schwartz J, *et al.,* (1988). Oral supplementations of Spirulina in humans prevented oral cancer. Spirulina was also reported to act as an antimutagen against Cyclophosphamide, Cisplatin and Mitomycin-C. Khan M, *et al.,* (2005).

Furthermore, its chemopreventive efficacy against skin tumors has been proved. Toxicological studies of several spirulina species have not revealed any toxic effect during and after different acute, chronic and reproductive tests. The antigenotoxic effect of Spirulina on the genotoxicity of two potent genotoxins Cisplatin and urethane has also been proved. Premkumar K, *et al.,* (2004)

Using micronucleus testing, it was shown that Spirulina significantly reduced both chromosomal damage and lipid peroxidation induced by Cyclophosphamide, mitomycin-C, Cisplatin and urethane in mice. Premkumar K, *et al.,* (2001); Premkumar K, *et al.,* (2004). When applied to bone marrow cells, the test revealed that Spirulina is radioprotective in mice. Qishen P, *et al.,* (1989) Finally, SP behaves as an anticlastogenic agent as shown by the Tradescantia bioassay. Ruiz-Flores LE, *et al.,* (2003)

In the present investigation, DOX was found to decrease the sperm count and increase the frequency of sperms with abnormal head. The sperm head abnormalities possibly result due to the interference with the DNA integrity and/or the expression of the genetic material. Administration of Spirulina prior to DOX was found to restore the sperm counts and decrease the incidence of sperms with head abnormalities.

Our results clearly demonstrate that intervention of Spirulina prior to DOX reduces the germ cell toxicity induced by it in rat as evident from the decreased sperm head abnormalities, and increased sperm counts.

Conclusion:

In conclusion, Spirulina was found to be a potential candidate as a protective agent to Doxorubicin-induced genotoxic effect in germ cells. The combined treatment of Doxorubicin and Spirulina holds promise as a safe and effective chemotherapeutic strategy.

Acknowledgement:

The authors are grateful to Dr.P.X.Antony M.V.Sc.,Ph.D. (Department of Veterinary microbiology), *Dr. C.R. Girija, M.V.Sc., Ph.D. (*Department of Animal Genetics & Breeding) and Dr. Kantharaj, M.V.Sc., (Department of Animal Reproduction, Gynaecology& Obstetrics), Rajiv Gandhi College of Veterinary and Animal Sciences, Puducherry for their assistance to do

the research. The authors also thankful to S. Arokianathan Ph.D., Dean (Subramania Bharathi School of Tamil Language & Literature) Dr. K. Srikumar Ph.D., FABMS., P. Muthuraman Ph.D. and Vikramathithan Ph.D., (Department of Biochemistry and Molecular Biology) Pondicherry University for their help to carry out the research work. The authors thank the authorities of Mother Theresa Postgraduate & Research institute of Health Sciences, Pondicherry Medical university for providing necessary facilities.

References:

1. Baumgartner A, Schmid TE, Cemeli E, Anderson D, (2004): Parallel evaluation of doxorubicin-induced genetic damage in human lymphocytes and sperm using the comet assay and spectral karyotyping, Mutagenesis, 19: 313-318.
2. Belay A, (2002): The potential application of Spirulina (*Arthrospira*) as a nutritional and therapeutic supplement in health management, Journal of the American Nutraceutical Association, 5: 26-48.
3. Bhat VB, Madyastha KM, (2001): Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from *Spirulina platensis*: protection against oxidative damage to DNA, Biochemical and Biophysical Research Communications, 285: 262–266.
4. Chamorro-Cevallos G, Gardun o-Siciliano L, Barron BL, Madrigal-Bujaidar E, Cruz-Vega DE, Pages N, (2008): Chemoprotective effect of Spirulina (*Arthrospira*) against cyclophosphamide-induced mutagenicity in mice, Food and Chemical Toxicology, 46: 567–574.
5. Choudhury RC, Ghosh SK, Palo AK, (2000): Cytogenetic toxicity of methotrexate in mouse bone marrow, Environmental Toxicology and Pharmacology, 8: 191–96.
6. Eastmond DA, Hartwig A, Anderson D, Anwar WA, Cimino MC, Dobrev I, Douglas GR, Nohmi T, Phillips DH, Vickers C, (2009): Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme, Mutagenesis, 24: 341-349.
7. Granados-Principal S, Quiles JL, Ramirez-Tortosa CL, Sanchez-Rovira P, Ramirez-Tortosa MC, (2010): New advances in molecular mechanisms and the prevention of Adriamycin toxicity by antioxidant nutrients, Food and Chemical Toxicology, 48:1425–1438.
8. http://faculty.ksu.edu.sa/73917/Documents/Papers/Mutagenicityof sometopoisomeraseIIinteractiveagents.pdf
9. Injac R, Strukelj B, (2008): Recent Advances in Protection Against Doxorubicin-induced Toxicity, Technology in Cancer Research and Treatment, 7:497-516.
10. Jahnukainen K, Hou M, Parvinen M, Eksborg S, Söder O, (2000): Stage-specific inhibition of deoxyribonucleic acid synthesis and induction of apoptosis by anthracyclines in cultured rat spermatogenic cells, Biology of Reproduction, 63: 482–487.
11. Kalender Y, Yel M, Kalender S, (2005). Doxorubicin hepatotoxicity and hepatic free radicals in rats. The effects of vitamin E and catechin, Toxicology, 209: 39–45.
12. Kang J, Lee Y, No K, Jung E, Sung J, Kimc Y, Nam S, (2002): Ginseng intestinal metabolite-I (GIM-I) reduces doxorubicin toxicity in mouse testis, Reproductive Toxicology, 16: 291–298.

13. Khan M, Shobha JC, Mohan IK, Rao Naidu MU, Sundaram C, Singh S, Kuppusamy P, Kutala VK, (2005): Protective Effect of Spirulina against Doxorubicin-induced Cardiotoxicity, Phytotherapy Research, 19: 1030–1037.
14. Miranda MS, Cintra RG, Barros SB, Mancini Filho J, (1998): Antioxidant activity of the microalga Spirulina maxima, The Brazilian Journal of Medical and Biological Research, 31: 1075–1079.
15. Padmanabhan S, Tripathi DN, Vikram A, Ramarao P, Jena GB, (2009). Methotrexate-induced cytotoxicity and genotoxicity in germ cells of mice: Intervention of folic and folinic acid. Mutation Research, 673: 43–52.
16. Prahalathan C, Selvakumar E, Varalakshmi P, (2006): Lipoic acid modulates adriamycin induced testicular toxicity, Reproductive Toxicology, 21: 54–59.
17. Prahalathan C, Selvekumar E, Varalakshmi P, (2005): Lipoic acid ameliorates Adriamycin induced testicular mitochondriopathy, Reproductive Toxicology, 20: 111–116.
18. Premkumar K, Abraham SK, Santhiya ST, Ramesh A, (2004): Protective effect of *Spirulina fusiformis* on chemical-induced genotoxicity in mice, Fitoterapia, 75: 24–31.
19. Premkumar K, Pachiappan A, Abraham SK, Santhiya ST, Gopinata PM, Ramesh A, (2001): Effect of *Spirulina fusiformis* on cyclophosphamide and mitomycin-C induced genotoxicity and oxidative stress in mice, Fitoterapia, 72: 906-911.
20. Qishen P, Baojiang G, Kolman A, (1989): Radioprotective effect of extract form *Spirulina platensis* in mouse bone marrow cell studied by using the micronucleus test. Toxicology Letters, 48: 165–169.
21. Quiles JL, Huertas JR, Battino M, Mataix J, Ramirez-Tortosa MC, (2002): Antioxidant nutrients and Adriamycin toxicity, Toxicology, 180:79-95.
22. Ray S, Roy K, Sengupta C, (2007): Evaluation of Protective Effects of Water Extract of *Spirulina platensis* (blue green algae) on Cisplatin - Induced Lipid Peroxidation, Indian Journal of Pharmaceutical Sciences,69: 378-383.
23. Romay C, González R, Ledón N, Remirez D, V Rimbau, (2003): C-Phycocyanin: A Biliprotein with Antioxidant, Anti-Inflammatory and Neuroprotective Effects, Current Protein & Peptide Science, 4: 207-216.
24. Ruiz-Flores LE, Madrigal-Bujaidar E, Salazar M, Chamorro G, (2003): Anticlastogenic effect of *Spirulina maxima* extract on the micronuclei induced by maleic hydrazide in Tradescantia, Life Science, 72: 1345–1351.
25. Sanocka D, Kurpisz M, (2004): Reactive oxygen species and sperm cells, Reproductive Biology and Endocrinology, 2: 1–7.
26. Schwartz J, Shklar G, Reid S, Trickler D, (1988): Prevention of experimental oral cancer by extracts of Spirulina-Dunaliella algae, Nutrition and Cancer, 11: 127–134.
27. Sikka SC, (1996): Oxidative stress and role of antioxidants in normal and abnormal sperm function, Frontiers in Bioscience, 1: 78–86.
28. Suominen JS, Linderborg J, Nikula H, Hakovirta H, Parvinen M, Toppari J, (2003): The effects of mono-2-ethylhexyl phathalate, adriamycin and N-ethyl-N-nitrosourea on stage-specific apoptosis and DNA synthesis in the mouse spermatogenesis. Toxicology Letters, 143: 63–73.
29. Xu X, Persson H L, Richardson D R, (2005): Molecular Pharmacology of the Interaction of Anthracyclines with Iron, Molecular Pharmacology, 68: 261–271.

ASHWAGANDHA: ROLE AS A WONDER THERAPEUTIC PLANT

Pratibha*[1,2], Ankit Yadav[1], Mayur Sonagara[1] and Unnati Vaghela[1]
[1]Anand Agricultural University, Anand, Gujarat, 388110
[2]Faculty of Agriculture, Madhav University, Abu Road, (Sirohi) Rajasthan-307026
*Corresponding author E-mail: taniya.2310@gmail.com

Abstract:

Ashwagandha is one of the most important therapeutic plants used in Indian medicine system since decades. It is a significant drug with the common name 'Asgand' that has been utilised in the Unani and Ayurvedic medical systems for centuries, either alone or in conjunction with other medications. It has also received a lot of recent scientific research due to its diverse medicinal possibilities. *Withania* is commonly believed to have powerful stimulant, relaxing, rejuvenative, and life-extension effects in Ayurveda. Additionally, it is employed for geriatric issues and as a general energy-boosting tonic called Medharasayana, which literally translates to "that which enhances learning and a good memory." Additionally, the species is employed in the treatment of Parkinson's disease and Alzheimer's disease. The two most renowned species of this genus, *Withania somnifera* and *Withania coagulans*, both have significant therapeutic uses. Withanolides (steroidal lactones), which are utilised as constituents in numerous formulations recommended for a wide range of disorders, are naturally produced by these species. The withanolides, are a class of naturally occurring C28-steroidal lactone triterpenoids supported by an intact or rearranged ergostane structure in which the carbon atoms C-22 and C-26 have undergone the proper oxidation to form a six-membered lactone ring. Potentially bioactive compounds include withaferinA, withanolide A, and withanolide D, which were isolated from this plant. This book chapter comprises of all the therapeutic and pharmaceutical uses of Ashwagandha plants till now.

Keywords: *Withania*, therapeutic, pharmaceutical, Ayurveda, withanolides

Introduction:

Plants have been utilised in traditional medicine for thousands of years and play a significant role in the discovery of new medicines (Muthu *et al.,* 2006). In Indian literature, plants are indeed a rich source of naturally occurring medicines with therapeutic value. These healing plants are in charge of making a variety of phytochemicals that give plants protection to disease. They have historically been a plentiful source of a wide range of lead compounds. To determine the existence of active components in medicinal plants, 95 phytochemical analysis is essential. Numerous medications have been discovered as a result of pharmacological testing of natural sources. Alkaloids, flavonoids, steroidal lactones, saponins, tannins, terpenoids, and other phytochemicals, generally known as secondary metabolites, have pharmacological and therapeutic properties like antibacterial activity and anti-inflammatory effects. Based on many medical systems like Ayurveda, Unani, and Siddha, the knowledge of medicinal plants has been acquired over many years.

About 84 genera and around 3,000 species that make up the Solanaceae family are dispersed throughout the globe (Mirjalili *et al.,* 2009). This family often consists of annual shrubs. *Withania*, a genus with 26 species worldwide and in India only two of this *Withania*

somnifera and *Withania coagulens* are mainly found. Due to its beneficial pharmacological and nutraceutical qualities, the genus *Withania* (Family: Solanaceae) is a highly regarded genus of medicinal plants in the Indian Ayurvedic school of medicine. *Withania* is a member of the tribe Physaleae, sub-tribe Withaninae, and subfamily Solanoideae of the Solanaceae family, of which it is the type genus (Olmstead *et al.*, 2008). *Withania* is mentioned in WHO monographs on Selected Medicinal Plants, and an upcoming monograph for the American Herbal Pharmacopoeia will also mention it (Marderosion *et al.*, 2001). It has also received a lot of recent scientific research due to its diverse medicinal possibilities. In Ayruveda, Siddha, and Unani medicine, ashwagandha roots are a component of over 200 formulations that are used to treat a variety of physiological ailments (Asthana and Raina, 1989; Singh and Kumar, 1998)

The plant has long been used to foster youth, endurance, strength, and health, as well as to nurture the body's temporal elements and boost the generation of cells, blood, lymph, and other essential bodily fluids. Additionally, it aids in preventing impotence, early ageing, emaciation, debility, chronic weariness, weakness, dehydration, weak bones and loose teeth, and muscle strain. The plant's leaves, which have a bitter flavour and are used as an antihelmantic. Fever is treated with the infusion (Nadkarni, 1976; Kapoor, 2001). Localized applications of bruised leaves and fruits are made to tumours, tubercular glands, carbuncles, and ulcers (Watt, 1972). The plant's smoke is inhaled to treat toothaches, and the twigs are chewed to clean teeth. Both as a vegetable and as cattle feed, the leaves are utilized (Kirtikar, 1991). It has been discovered that the plant's unprocessed form is effective against a variety of harmful microorganisms (Khan, 1993). The pulp and husk of the berry from *Withania coagulens*, which have been employed in the creation of vegetable rennet ferment for cheese, are said to have a milk-coagulating activity that is attributed to the plant's fruits (Atal, 1963). An enzyme is responsible for the milk-coagulating action, and under ideal circumstances, one part of concentrated enzyme can coagulate 90,000 parts of milk in 30 minutes. The fruits are said to have sedative, emetic, and stomachic properties, as well as being a blood purifier, febrifuge, alterative, diuretic, and bitter tonic in cases of dyspepsia, as well as a growth promoter in young children (Singh & Kumar, 1998). In pregnant women and the elderly, the roots are used as a nutrient and health tonic. For the treatment of female infertility, the decoction of the root is advised. It is boiled with milk and ghee. Additionally, the roots are used to treat spermatorrhoea, senile debility, rheumatism, general debility, nervous weariness, memory loss, and rheumatism (Watt, 1972).

Distribution

The plant is widely distributed geographically and is mostly adapted to the drier, warmer areas of tropical and subtropical regions around the world, including Southwest Asia, tropical Africa, South Africa, and Mediterranean regions. It is indigenous to Tropical Asia, particularly India, Pakistan, and Sri Lanka. *W. somnifera* is currently grown extensively in the drier regions of India, specifically in the Madhya Pradesh, Punjab, Gujarat, and Rajasthan.

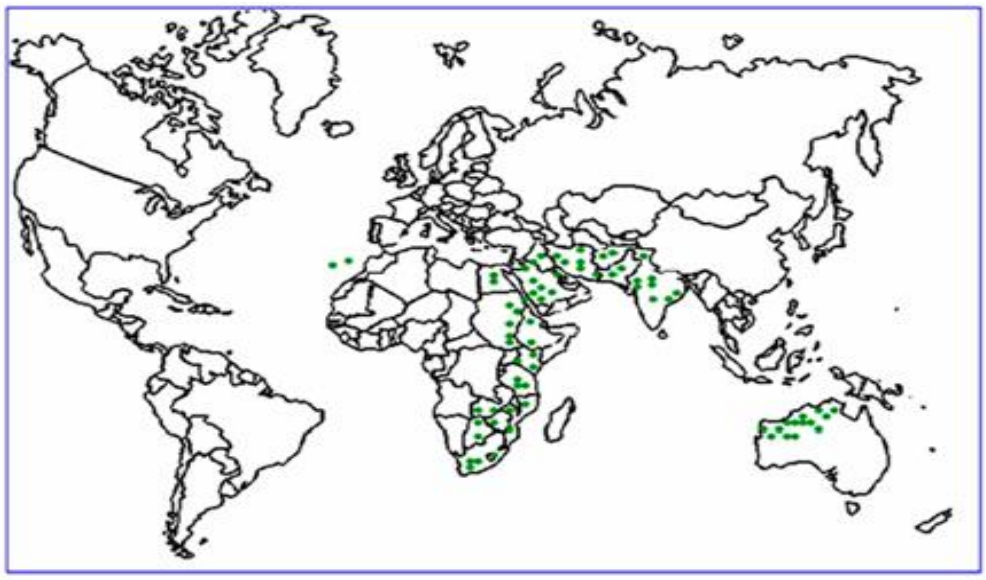


Figure 1: World distribution of *Withania somnifera* (Credit: Mir *et al.*, 2012)

Morphology

Withania somnifera is a 30-150 cm tall, tomentose, upright, evergreen shrub. Simple, oval, glabrous, petiolate leaves have a leaf blade that is elliptic-ovate to broadly ovate in shape, entire along the margins, acute to obtuse at the apex, cuneate or oblique at the base, persistently covered in whitish tomentum on the sides, and up to 10 cm long leaves are present. Few flowers (often around 5) are born together in axillary, umbellate cymes, and they are little, approximately 1 cm long, greenish or bright yellow blooms (short axillary clusters). It produces blooms all year round, with a peak flowering period between March and July. Due to the filaments extension to cover the bilobed stigmatic surface with dehiscing anthers, the species displays stigma-anther closeness. When fully grown, the fruits are 6 mm in diameter, orange red in colour, and encased in an inflated, persistent calyx with membranes. Yellow, reniform seeds are 2.5 mm in diameter (Anonymous, 2007). Self-pollination is significantly favoured by a high pollen load on the stigma and fierce pollen competition within a flower (Mir *et al.*, 2012). The species has been noted to exhibit polysomatomy (2n=12, 2n=18, 2n=24, 2n=36, 2n=48 and 2n=72) with a majority of the 2n=48 type, as well as diploid (2n = 24), tetraploid (2n = 48), and hexaploid (2n = 72) cytotypes (Iqbal and Dutta, 2007).

Chemical composition

Secondary metabolites are produced by a number of distinct geographically and temporally distributed processes in medicinal plants. Alterations to the environment or growing a plant in tissue culture can both result in the creation of a secondary metabolic profile that is novel and occasionally unexpected. Phytochemical composition of *W. somnifera* has undergone substantial research. Power and Salway (1911), who isolated an amorphous alkaloid ($C_{12}H_{16}N_2$) from a South African strain of *W. somnifera*, marked the beginning of chemical characterisation. Later, Majumdar and Guha (1933) examined into a plant of *W. somnifera* from Bengal and discovered the alkaloid. Later, they claimed that the roots contained nicotine as well as seven other alkaloids that they named without providing any structural details: somniferine, somniferinine, somnine, withamine, pseudowithamine, withanmine, and withanaminine (Majumdar, 1955). Currently, aerial portions, roots, and berries of *Withania* species have provided more than 13 alkaloids, 138 withanolides, and many sitoindosides (a withanolide containing a glucose molecule at carbon 27). (Subramanian and Sethi, 1969, 1971; Velde *et al.*, 1983; Atta-ur-Rahman *et al.*, 1993; Atta-ur-Rahman *et al.*, 1998, 1999, 2003; Mirjalili *et al.*, 2009b; Xu *et al.*, 2011). Withanolides, the primary chemical components of this plant, are primarily concentrated in the leaves and roots and range in concentration from 0.001 to 0.5% dry weight (Kapoor, 2001). The withanolides are a class of C28-steroidal lactones that are based on an ergostane structure with a six-membered lactone ring formed by the oxidation of C-22 and C-26. The "withanolide skeleton" is the name given to the fundamental structure (Tursunova *et al.*, 1977; Glotter, 1991; Alfonso *et al.*, 1993).

Not all Solanaceae family members have this class of chemicals. One definition of the withanolide skeleton is a 22-hydroxyergostan-26-oic acid-26, 22-lactone. Many unique structures containing withanolides can be created by changing the side chain or the carbocyclic skeleton. According to some reports, plants that accumulate these polyoxygenated compounds are equipped with machinery that can oxidise every carbon atom in the steroid nucleus. Though withanolides are the main bioactive substances present in both species, some withanolides are unique to each. While coagulin L has been discovered in significant proportions in *W. coagulans*, withaferin A is a prominent component found in *W. somnifera*.

Withaferin A (4, 27-dihydroxyl-1-oxo-5, 6-epoxywitha-2-24-dienolide) was the first substance in this class to be isolated from a South Asian strain, and Lavie *et al* (1965). have now deduced its structure for the first time. Numerous compounds with similar structural characteristics were identified as a result of a thorough chemical examination of the plant in response to the unexpected structural properties and intriguing biological actions this substance evoked (Glotter, 1991). From this amazing medicinal plant, some significant bioactive compounds were identified that may be used in the creation of new drugs. The investigation of the roots of *W. somnifera* led to the discovery of ashwagandhanolide, a novel dimeric thiowithanolide (Subaraju *et al.*, 2006; Mirjallili *et al.*, 2009).

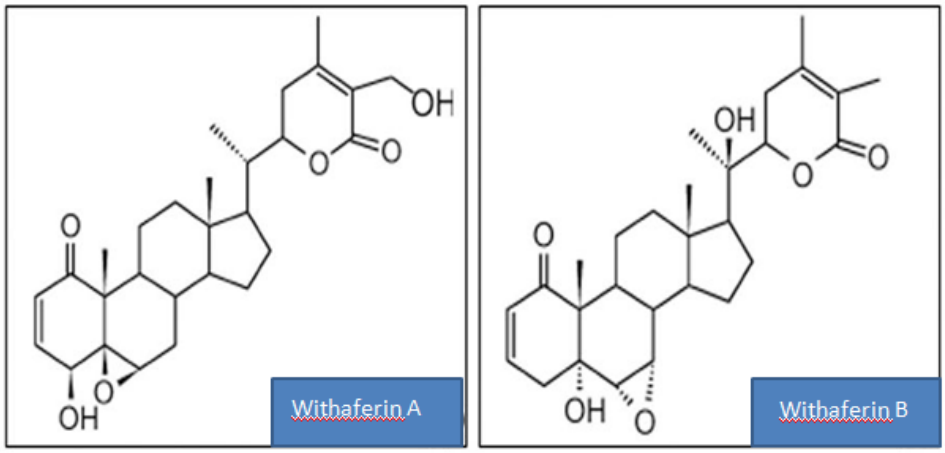


Figure 2: Structures of important withanolides

There have also been reports of numerous more chemical components in *W. somnifera* and *W. coagulans*. For instance, all plant components (roots, fruits, and leaves) were discovered to contain alkaloids according to Gupta *et al.* (1996), with leaves having the highest concentration. Another investigation found this therapeutic plant to include nicotine, somniferine, somniferinine, withanine, withananine, pseudowithanine, tropine, pseudotropine, 3a-tigloyloxy-tropane, choline, cuscohygrine, dl-isopelletierine, and the novel alkaloids anaferine and anhygrine (Gupta and Rana, 2007). The range for the total alkaloid content was 0.13 to 0.31% (Johri *et al.,* 2005). The plant also includes a high amount of iron, acylsteryl glucosides, starch, hantrea-cotane, ducitol, and a variety of amino acids such as aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cystine, and tryptophan (Gupta and Rana, 2007; Hemalatha *et al.,* 2008). Gupta *et al.* (1996) identified alkaloids in all the aforementioned plant parts during their quantitative examination of Indian chemotypes of *W. somnifera* using TLC densitometry, with the leaves having the highest concentration. Contrary to popular perception, tropane alkaloids are not only found in the roots of *Withania* spp. The most alkaloids are produced when 45% alcohol is used in the extraction process. Withanine, withananine, pseudowithanine, tropine, pseudotropine, 3-tigloyloxytropane, choline, cuscohygrine, dl-isopelletierine, and the newly discovered alkaloids anaferine and anhygrine have all been isolated (Kapoor, 2001), (Gupta, 2007) The leaves are said to contain chlorogenic acid, calystegines (nitrogen-containing polyhydroxylated heterocyclic compounds), withanone, condensed tannin, and flavonoids, with a yield of five unidentified alkaloids (yield 0.09%). Amino acids can be found in berries. From the roots of *W. somnifera*, four different types of peroxidases have been isolated and identified. (Kapoor, 2001, Johri, S. *et al.* 2005).

Therapeutic uses

Pharmocological profile of ashwagandha

Gupta and Rana (2007) have provided a summary of the pharmacological effects of *W. somnifera* preparations. More recently, *W. somnifera* has been used as an antibiotic, antihyperplycemic, antitumoral, aphrodisiac, liver tonic, anti-inflammatory agent, and astringent, as well as to treat ulcers and senile dementia. Some of the major activities of *W. somnifera* as its pharmacology are mentioned under various subheads.

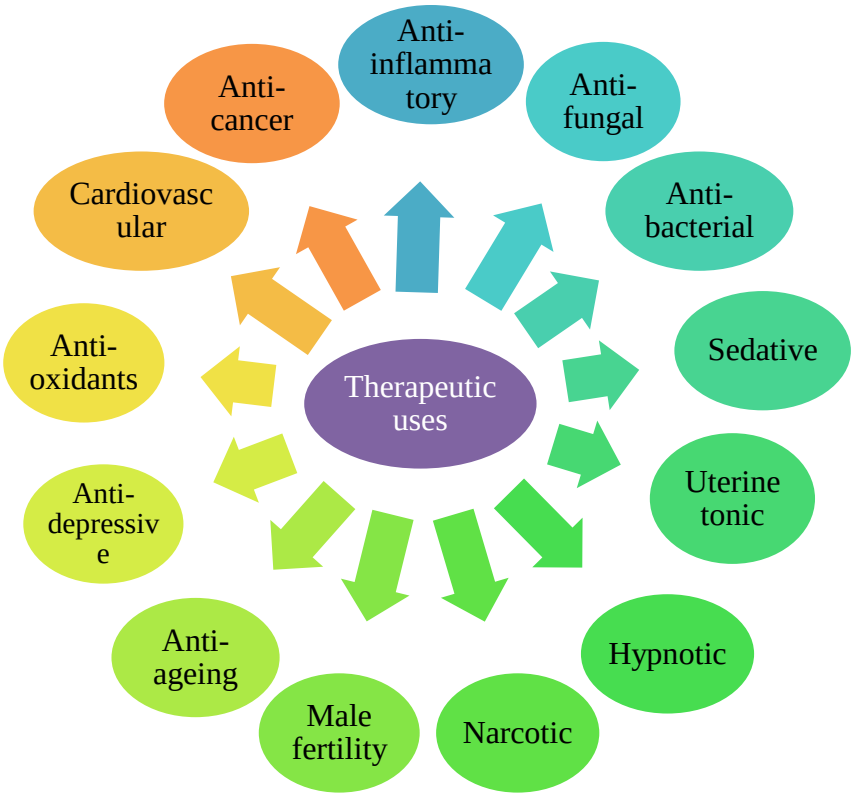


Figure 3: Showing therapeutic uses of Ashwagandha

Antifungal and antibacterial activities

The withanolides that were extracted from the ethanolic extract of the whole plant and the leaves, respectively, have been shown to exhibit antifungal and antibacterial effects. Maximum inhibitory activity was present in the methanolic extract against a variety of microorganisms. *Salmonella* infection in mice was efficiently eradicated by oral administration of the aqueous fruit extracts, as demonstrated by improved survival rates and decreased bacterial loads in numerous important organs of the treated animals. Agar plate disc-diffusion assays against *Salmonella typhimurium* and *Escherichia coli* were used to test the antibacterial and synergistic activity of methanol, hexane, and diethyl ether extracts from the leaves and roots of *W. somnifera.*

Anti-inflammatory activities

Through the inhibition of complement, lymphocyte proliferation, and delayed-type hypersensitivity, ashwagandha serves as an anti-inflammatory drug. *W. somnifera* extracts have demonstrated anti-inflammatory benefits in a number of rheumatological diseases (Anbalagan *et al.,* 1981). It was discovered that the extract significantly reduced the amount of glycosaminoglycans in the granuloma tissue and uncoupled oxidative phosphorylation by significantly lowering the ADP/O ratio in the mitochondria of the granuloma tissue. It also increased the activity of the Mg^{2+} dependent-ATPase enzyme, which led to a decrease in succinate dehydrogenase activity in the mitochondria of the granuloma tissue.

a2-macroglobulin, a marker for anti-inflammatory medicines, was suppressed by *W. somnifera* in rat serum that had been inflamed by injection of carrageenan suspension in another investigation (Anbalagan *et al.,* 1984). Additionally, as seen by radiographic inspection, the

extracts significantly reduced the paw edoema and bone degenerative changes in Freund's adjuvant-induced arthritis (Begum *et al.,* 1988) Rats receiving formaline injections in the footpad of the hind leg displayed decreased 14C-glucose absorption in the rat jejunum; however, *Withania* extracts, which had an anti-inflammatory action, kept glucose absorption at the normal level (Somasundaram *et al.,* 1983).

Anticancer activity

According to reports, withanolides A and D have considerable anti-tumor and radiosensitizing properties (Lyon and Kuttan, 2004). Another component of *W. somnifera*, 1-oxo-5ß, 6-epoxy-witha-2-enolide, has been linked to a decrease in skin cancer brought on by UV radiation. When human larynx carcinoma cells are cultivated, withaferin A functions as a mitotic toxin, stopping cell division at metaphase (Mathur *et al.,* 2004). Additionally, it resulted in a sizable dose-dependent inhibition of Ehrlich ascites carcinoma, sarcoma 180, sarcoma Black, and E 0771 mammary adenocarcinoma growth (Davis and Kuttan, 1998). The multiplication of stem cells has been aided by *W. somnifera* methanolic extract (Kuttan, 1996). It also reduced the viability of breast, lung, central nervous system, and colon cancer cell lines in a sleep-dependent manner, which suggests that it has potential as a chemotherapeutic drug (Jayaprakasan *et al.,*2003). The apoptosis induction defined by DNA condensation, cytoplasmic histone-associated DNA fragmentation, and cleavage of poly-(ADP-ribose)-polymerase was connected with the withaferin A-mediated lowering of breast cancer cell survival.

Antioxidant effects

The levels of the main free-radical scavenging enzymes, superoxide dismutase, catalase, and glutathione peroxidase, in the rat brain frontal cortex and striatum have been used to investigate the antioxidant activity of *W. somnifera*'s active ingredients. The rise in these enzymes following withanolide administration indicates increased antioxidant activity and a commensurate protective impact on neural tissue, which raises the possibility that *W. somnifera*'s antioxidant effect in the brain underlies its varied pharmacological effects (Bhattacharya *et al.,* 1997). Similarly, oral administration of *W. somnifera* extracts stopped mice and rabbits from experiencing an increase in lipid peroxidation (Dhuley *et al.,* 1998).

The brain and nervous system are relatively more vulnerable to free radical damage than other tissues because they contain high levels of lipids and iron, both of which are known to encourage the production of reactive oxygen species (Halliwell & Gutteridge, 1989). In addition to being a factor in ageing and neurodegenerative diseases like Parkinson's, Alzheimer's, epilepsy, and schizophrenia, free radical damage to nervous tissue may also be the cause of neural loss in cerebral ischemia (Sehgal *et al.,* 2012). The active tenets of Withaferin A (glycowithanolides), sitoindosides VII–X, and somnifera are said to increase endogenous superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and ascorbic acid levels while simultaneously lowering lipid peroxidation (Bhattacharya, 2001). It is known that a drop in these enzymes' activity can cause an accumulation of oxidative free radicals, which can have degenerative effects.

Antistress and Aphrodisiac activity

Rats have been used to test the bioactive withanolides' anxiolytic and depressive effects. (Bhattacharya *et al.,* 2000). When pentylenetetrazole, an anxiogenic drug, increased the levels of

tribulin (an endocoid marker of anxiety), withanolides decreased the amounts of this substance in the rat brain. In the forced swim-induced "behavioural despair" and "learned helpessness" test, withanolides had an antidepressant effect comparable to that generated by imipramine. Withanolides were able to reverse chronic stress-induced restriction of male sexual behaviour, reduce the number and severity of chronic stress-induced ulcers, boost peritoneal macrophage activity, and reverse chronic stress-induced immunosuppression in a rat model (Bhattacharya *et al.,* 2003). Additionally, methanolic extracts of *withania* have been shown to lower the ulcer index, volume of stomach secretion, free acidity, and total acidity (Bhatnagar *et al.,* 2005). Men who used ashwagandha experienced more vigour performance and it is also utilised as a tonic in the treatment of spermatopathia, impotence, and seminal depletion (Nadkarni, 1976). The increased concentrations of inorganic elements like Fe, Mg, K and Ni in the roots of this plant play a vital part in the diuretic and aphrodisiac activity of the medicine (Lohar *et al.,* 1992). The decoction of the root boiled with milk and ghee is recommended for curing sterility in women (Singh & Kumar, 1998).

According to reports, ashwagandha has a calming rather than a stimulative effect on the central nervous system, making it a preferable treatment for nervous irritation and fatigue. Neurotransmitters known to play a significant part in brain functions including memory are known to have their concentrations altered by ashwagandha. Ashwagandholine's effects on the neurological system are related (root extracts). It causes relaxant and antispasmodic effects against numerous drugs that elicit smooth muscle spasms in intestinal, uterine, tracheal, and vascular muscles, and it enhances barbiturate-, ethanol-, and urethane-induced hypnosis in mice (Malhotra *et al.,* 1965). According to reports, the bioactive substances mainly affect the cholinergic-signal transduction cascade events in the cortical and basal forebrain.

Hepatoprotective activity

Due to the presence of 3-hydroxy-2, 3-dihydro-withanolide F, the root extract of *W. coagulans* showed hepato-protective efficacy against carbon tetrachloride (CCl4)-induced hepatotoxicity in adult albino rats of either sex. Mohanty *et al.* (2008) investigated the hepatoprotective effects of *W. somnifera* root powder. Hepatoprotection was provided by the extract through altering the levels of lipid peroxidation. Verma *et al.* (2009) investigated how *W. somnifera* aqueous root extract affected the hepatic cell of *Clarias batrachus*, and they found that the root extract contained various flavonoids and neurotransmitters that stimulated the neuroendocrine system, causing the endomembrane to become hyperactive and molecules to exocytose out of the surface (Jain *et al.,*2012).

Cardiovascular Protection

In hypercholesterolemic rats, the aqueous extract of *W. coagulans* fruit and the root powder of *W. somnifera* have been shown to lower total lipid, cholesterol, and triglycerides Hemalatha *et al.,* 2006. In a study on the hypocholesterolemic action of *W. somnifera* in male albino rats, Visavadiya and Narasimhacharya (2007) hypothesised that the hypocholesterolemic impact of *W. somnifera* would be mediated by an enhanced bile acid production for the removal of body cholesterol. Hemalatha *et al.* also reported on the hypocholesterolemic and hypolipidemic properties of *W. coagulans* (2006). After giving high-fat diet-induced hyperlipidemic mice an aqueous extract of the fruits of *W. coagulans* for seven weeks, the levels

of increased blood cholesterol, triglycerides, and lipoprotein were dramatically lowered. Additionally, in triton-induced hypercholesterolemia, this extract demonstrated hypolipidemic action (Hemalatha *et al.*, 2006).

Table 1: List of ashwagandha plant part used for Cardiovascular protection in different medicine system

System of medicine	Uses	Plant Part	References
Ayurveda	Rejuvenating drug, tonic, Alternative pungent, astringent, Aphrodisiac, Phthisis	Roots	Dutta (1877), Kumar *et al.*, (1980), Sen Gupta (1984)
	Aphrodisiac, carbuncle, Ulcers, painful swelling	Leaves	Dutta (1877), Kumar *et al.*, (1980), Singh and Kumar (1998), Mhaskar *et al.*, (2000)
	Diuretic, narcotic and hypnotic	Seeds	Dalzell and Gibson (1861)
	Antihelmantic, ulcers and tubercular glands	Fruits	Nadkarni (1976), Kapoor (2001)
Siddha	Aphrodisiac, fever, inflammation	Roots	SPC (1992)
	Fever, chest pain, sores, swelling	Leaves	SPC (1992)
Unani	Asthma, bronchitis, leucoderma, Arthritis, emenagogue	Roots	Stewart (1869), Mathani (1973)
	External pains, anti-inflammatory	Leaves	UPC (1993)
Folklare	Abortificiant, cold, asthma, Tuberculosis, fever	Roots	Dutta (1877), Kumar *et al.*, (1980), Singh and Kumar (1998)
	Cure eyesores, boils, diuretic Narcotic, treatment of syphilis and hemorrhoids	Leaves	Shah and Gopal (1985), Sharma *et al.*, (1992)
	To coagulate milk, Applied on open wounds, Relieving the poison of a serpent rubbed on skin for ringworm in human beings and animals	Seeds	Dalzell and Gibson (1861), Rao (1977), Sahu (1982), Shah and Gopal (1985), Dafni and Yaniv (1994)

Immunity

By increasing the levels of interferon (IFN)-, interleukin (IL)-2, and granulocyte macrophage colony stimulating factor in healthy and cyclophosphamide-treated mice, *W.*

somnifera exhibits immuno-potentiating and myeloprotective properties (Davis & Kuttan, 1999). The plant helps to increase red blood cell count since it is high in iron. Rather than merely reducing the immunological/inflammatory response, *W. somnifera* has a more subtle impact on the immune system. The active ingredient (withanolide A) in the roots of *W. somnifera* dramatically raises CD4 and CD8 counts as well as the expression levels of T-helper 1 (Th1) cytokines. Additionally, it speeds up the recovery of CD4+ T cells in immunological suppressed mice while enhancing natural killer (NK) cell activity in a dose-dependent manner (Khan *et al.*, 2006). In addition to the previously mentioned elevated nitrile, IL-2, and TNF-2 secretion, mild IL-4 decreases, with no impact on IL-10, suggesting that it solely affected the Th1 profile of the cytokines. According to lymphocyte surface markers on T cells (CD3+, CD4+, and CD8+) and B cells (CD19+), the root powder of this plant is also said to activate cell-mediated immunity, IgM and IgG as well as a notable improvement in lymphocyte proliferation and differentiation (Singh *et al.*, 2001).

Anti-ageing Effect

In a clinical investigation, 101 healthy, normal guys between the ages of 50 and 59 participated in a double-blind to examine the effects of plants on the prevention of ageing. For a year, root powder (0.5 g) was administered orally three times per day. In compared to the placebo group, the treated group's Hb, RBC, hair melanin, and sitting height all increased statistically significantly. The treatment group experienced a greater drop in serum cholesterol than the placebo group (Rastogi *et al.*, 1998).

According to reports, *W. somnifera* has a growth-promoting impact either taken alone in powder form or in combination with other medications. Withanolides are blamed for the activity that promotes growth (Budhiraja & Sudhir, 1987). The study found significant improvements in haemoglobin, packed cell volume, mean corpuscular volume, serum iron, body weight, hand grip, and total proteins in both children and elderly participants. In adults, serum cholesterol fell while nail calcium was maintained. 71.4 percent of them reported greater vigour, and erythrocyte sedimentation rate dramatically lowered (Kuppurajan *et al.*, 1980). In conclusion, these investigations suggest that *W. somnifera* may be effective as a general health tonic in both younger and older people.

Conclusion:

Ashwagandha, which has been used for centuries to cure a variety of disorders and is chemically rich with its varied content of active chemicals including withanolides, sitoindosides, and many beneficial alkaloids, represents a viable contender as a multi-purpose medicinal agent. Because they have fewer or no adverse effects compared to synthetic pharmaceuticals, herbal drugs are becoming more and more popular throughout the world. Different plants are said to have therapeutic properties in Ayurveda. This plant that produces multiple types of drugs has undergone a lot of development up to this point. *W. somnifera* and *W. coagulans* are the two important species of the *Withania* genus which are found in India. The main constituents present in both the species are withanolides, some of which are unique to each species. While coagulin L has been discovered in significant proportions in *W. coagulans*, withaferin A is a prominent component found in *W. somnifera*. Ashwagandhanolide is a special thio-dimer of withanolide that has been discovered in *W. somnifera*. Withanolides might take on a significant role in the

world. The range of reported activity for the withanolides, fractions, and extracts obtained from this miraculous medicinal plant offer encouraging support for further study.

References:

1. Alfonso D, Bernardinelli G, Kapetanidis I (1993). Withanolides from *Lochroma coccineum*. *Phytochemistry* 34:517-521.
2. Anbalagan K and Sadique J (1984) Role of prostaglandins in acute phase proteins in inflammation. *Biochem. Med*. 31, 236-245.
3. Anbalagan K, Sadique J (1981). Influence of an Indian medicine (Ashwagandha) on acute-phase reactants in inflammation. *Indian J. Exp. Biol.* 19:245-249.
4. Anonymous. The Unani Pharmacopoeia of India. Part I, Vol. I, Depatt. of AYUSH, Ministry of Health & Family Welfare, Govt. of India, New Delhi (2007) 7-8.
5. Asthana R and Raina MK (1989) Pharmacology of *Withania somnifera* (L.) Dunal-a review. *Ind Drugs*. 26, 199-205.
6. Atal, C.K.; Sethi, P.D. A preliminary chemical examination of *Withania coagulans*. *Indian J.*
7. Atta-ur-Rahman A, Abbas S, Dur-e-Shawar NA, Jamal AS, Choudhary MI (1993). New withanolides from *Withania* spp. 56, 1000-1006. *J. Nat. Prod.* 56:1000-1006.
8. Atta-ur-Rahman A, Choudhary MI, Qureshi S, Gul W, Yousaf M (1998). Two new ergostane-type steroidal lactones from *Withania coagulans*. *J. Nat. Prod.* 61:812-814.
9. Atta-ur-Rahman A, Dur-e-Shahwar NA, Choudhary MI (2003). Withanolides from *Withania coagulans*. *Phytochemistry* 63:387-390.
10. Atta-ur-Rahman A, Shabbir M, Yousaf M, Qureshi S, Dur-e-Shahwar NA, Naz A, Choudhary MI (1999). Three withanolides from *Withania coagulans*. *Phytochemistry* 52:1361-1364.
11. Begum VH and Sadique J (1988) Long term effect of herbal drug *Withania somnifera* on adjuvant induced arthritis in rats. *Ind. J. Exp. Biol.* 26, 877-882.
12. Begum VH, Sadique J (1987). Effect of *Withania somnifera* on glycosaminoglycan synthesis in carrageenin-induced air pouch granuloma. *Biochem Med. Metab. Biol*. 38:272-277.
13. Bhatnagar M, Sisodia SS and Bhatnagar R (2005) Antiulcer and antioxidant activity of *Asparagus racemosus* WILLD and *Withania somnifera* DUNAL in Rats. *Ann. NY Acad. Sci.* 1056, 261-278.
14. Bhattacharya A, Ghosal S and Bhattacharya SK (2001) Antioxidant effect of *Withania somnifera* glycowithanolides in chronic footshock stress-induced perturbations of oxidative free radical scavenging enzymes and lipid peroxidation in rat frontal cortex and striatum. *J. Ethnopharmacol*. 74: 1-6.
15. Bhattacharya SK and Muruganandam AV (2003) Adaptogenic activity of *Withania somnifera*: an experimental study using a rat model of chronic stress. *Pharmacol. Biochem. Behav*. 75, 547-555.
16. Bhattacharya SK, Bhattacharya A, Sairam K and Ghosal S (2000) Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. *Phytomed.* 7, 463-469.

17. Bhattacharya SK, Goel RK, Kaur R and Ghosal S (1987) Antistress activity of sitoindosides VII and VIII, new acylsterylglucosides from *Withania somnifera*. *Phytother. Res*. 1, 32-39.
18. Budhiraja RD and Sudhir S (1987) Review of biological activity of withanolides. *J. Sci. Ind. Res.* 1987, 46, 488-491.
19. Davis L and Kuttan G (1998) Suppressive: Effect of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice. *J. Ethnopharmacol.* 62, 209-214.
20. Davis L and Kuttan G (1998) Suppressive: Effect of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice. *J. Ethnopharmacol.* 62, 209-214.
21. Dhuley JN (1998) Effect of ashwagandha on lipid peroxidation in stress-induced animals. *J Ethnopharmacol.* 60, 173- 178
22. Glotter E (1991). Withanolides and related ergostane-type steroids. Nat. Prod. Rep. 8:415-440.
23. Gupta AP, Verma RK, Misra HO, Gupta MM (1996). Quantitative determination of withaferin A in different plant parts of *Withania somnifera* by TLC densitometry. *J. Med. Aromat. Plant Sci.* 18:788-790.
24. Gupta GL, Rana AC (2007). *Withania somnifera* (Ashwagandha): A review. *Phcog Rev.* 1:129-136.
25. Halliwell B and Gutteridge (1989) JMC Free radicals in biology & medicine. 2nd ed. Oxford: clarendon press.
26. Hemalatha S, Kumar R, Kumar M (2008). *Withania coagulans* Dunal: A review. Phcog Rev. 2:351-358.
27. Hemalatha S, Wahi AK, Singh PN, Chansouria JPN (2006). Hypolipidemic activity of aqueous extract of *Withania coagulans* Dunal in albino rats. *Phytother. Res*. 20:614-617.
28. Iqbal, M. & Datta, A. K. (2007). Genetic variability, correlation and path analysis in *Withania somnifera* (L.) Dunal (Ashwagandha). *Journal of Phytological Research,* 20 (1), 119 - 122.
29. Kuttan G (1996) Use of *Withania somnifera* Dunal as an adjuvant during radiation therapy. *Ind. J. Exp. Biol.* 34, 854-856.
30. Jain, R., Kachhwaha, S., & Kothari, S. L. (2012). Phytochemistry, pharmacology, and biotechnology of *Withania somnifera* and *Withania coagulans*: A review. *Journal of Medicinal Plants Research*, *6*(41), 5388-5399.
31. Jayaprakasam B, Zhang Y. Seeram NP and Nair MG (2003) Growth inhibition of human tumor cell lines by withanolides from *Withania somnifera* leaves. *Life Sci.* 2003, 74, 125-132.
32. Johri S, Jamwal U, Rasool S, Kumar A, Verma V, Qazi GN (2005). Purification and characterization of peroxidases from *Withania somnifera* (AGB 002) and their ability to oxidize IAA. *Plant Sci*. 169:1014-1021.
33. Kapoor LD (2001). Handbook of Ayurvedic medicinal plants. CRC Press
34. Khan B, Ahmad SF and Bani S *et al.*, (2006) Augmentation and proliferation of T lymphocytes and Th-1 cytokines by *Withania somnifera* in stressed mice. *Int. Immunopharmacol.* 6, 1394-1403.

35. Khan, M.T.J.; Ashraf, M.; Tehniyat, S.; Bukhtair, M.K.; Ashraf, S.; Ahmad W. Antibacterial activity of *Withania coagulans*. *Fitoterapia* 1993, *64*, 367-370.
36. Kirtikar, K. R. (1991). Indian Medicinal Plants. *(No Title)*.
37. Kuppurajan K et al,. J Res Ayu Sid 1, 247, 1980. As cited in Bone K. Clinical Applications of Ayurvedic and Chinese Herbs. Monographs for the Western Herbal Practitioner. Australia: *Phytotherapy Press*; 1996:137-141
38. Lavie, D., Glotter, E.; Shvo, Y. Constituents of *Withania somnifera* Dun. Part IV The structure of withaferin-A. *J. Am. Chem. Soc*. 1965, *30*, 7517-7531.
39. Leyon PV and Kuttan G (2004) Effect of *Withania somnifera* on B16F-10 melanoma induced metastasis in mice. *Phytother. Res.* 18, 118-122.
40. Lohar DR, Chaturvedi D and Varma PN (1992) Mineral elements of a few medicinally important plants. *Ind. Drugs*. 29, 271-273.
41. Majumdar, D. N., & Guha, P. C. (1933). Indian medicinal plants. Part I. Withania somnifera. *Journal of the Indian Institute of Science*, *16*, 29.
42. Majumdar, R. C. (1955). *An Advanced history of India Pt.-1*. Macmillan.
43. Malhotra CL, Mehta VL, Das PK and Dhalla NS (1965) Studies on *Withania-ashwagandha*, Kaul. V. The effect of total alkaloids (ashwagandholine) on the central nervous system. *Ind. J. Physio .Pharmacol.* 9, 127-136.
44. Marderosion, A. D. (2001). The Review of Natural Products, Facts and Comparisons. *St. Louis, MI, USA*, 630-632.
45. Mathur S, Kaur P and Sharma M *et al* (2004) The treatment of skin carcinoma induced by UV B radiation, using 1-oxo-5beta, 6beta -epoxy-with a-2-enolide, isolated from the roots of *Withania somnifera*, in a rat model. *Phytomed.* 11, 452-460
46. Mir BA, Khazir J, Mir NA, Dar TH, Koul S (2012) Botanical, chemical and pharmacological review of *Withania somnifera* (Indian ginseng): an ayurvedic medicinal plant. *Ind J Drugs Diseases* 6:147–160
47. Mirjalili HM, Fakhr-Tabatabaei SM, Bonfill M, Alizadeh H, Cusido RM, Ghassempour A, Palazon J (2009a). Morphology and withanolide production of *Withania coagulans* hairy root cultures. *Eng. Life Sci*. 9:197-204.
48. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazon J (2009b). Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. *Molecules* 14:2373-2393.
49. Mohanty I, Arya DS, Dinda A, Talwar KK, Joshi S and Gupta SK (2004) Mechanisms of Cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. *Basic clin. Pharmacol. Toxicol.* 94 (4), 184-190.
50. Mohanty IR, Arya DS, Gupta SK (2008). *Withania somnifera* provides cardioprotection and attenuates ischemia-reperfusion induced apoptosis. *Clin. Nutr.* 27:635-642.
51. Muthu, C., Ayyanar, M., Raja, N., & Ignacimuthu, S. (2006). Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, India. *Journal of Ethnobiology and ethnomedicine*, *2*(1), 1-10.
52. Nadkarni, K.M., 1976. Indian Materia Medica, Vol. I & II. Popular Prakashan Private Limited, Bombay, India.

53. Olmstead, R. G., Bohs, L., Migid, H. A., Santiago-Valentin, E., Garcia, V. F., & Collier, S. M. (2008). A molecular phylogeny of the Solanaceae. Taxon, 57(4), 1159-1181. *Pharm.* 1963, *25*, 163-164.
54. Power, F. B. and A. H. Salway. The constituents of *Withania somnifera.* Jour. Chem. Soc.99: 490. 1911.
55. Rastogi RP and Mehrotra BN. Compendium of Indian Medicinal Plants. 2nd Reprint, Central Drug Research Institute, Lucknow and National Institute of Science Communication, Council of Scientific and Industrial Research, New Delhi (1998) Vol. 1: 434-436; Vol. 2: 708-710; Vol. 3: 682-684; Vol. 4: 765-766; Vol. 5: 889-891; Vol. 6: 148.
56. Sehgal N, Gupta A, Khader R, Shanker V, Joshi D, Mills JT, Hamel E, Khanna P, Jain SC, Thakur SS, and Ravindranath V (2012) *Withania somnifera* reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver. *Proc. Nat. Acad. Sci.* 109(9), 3510-3515.
57. Singh B, Saxena AK, Chandan BK, Gupta DK, Bhutani KK and Anand KK (2001) Adaptogenic activity of a novel, withanolide-free aqueous fraction from the roots of *Withania somnifera* Dun. *Phytother. Res*. 15 (4), 311-318.
58. Singh S and Kumar S (1998) *Withania somnifera*: The Indian Ginseng Ashwagandha, Central Institute of Medicinal and Aromatic Plants: Lucknow, India. Somasundaram, S.; Sadique, J.; Subramoniam, A. Influence of extra-intestinal inflammation on the in vitro absorption of 14C-glucose and the effects of anti-inflammatory drugs in the jejunum of rats. *Clin. Exp. Pharmacol. Physiol.* 1983, 10, 147-152.
59. Subramanian, S. S., & Sethi, P. D. (1969). Withaferin-A from the roots of *Withania coagulans*. *Current Science*, *38*(11), 267-268.
60. Subramanian, S. S., Sethi, P. D., Glotter, E., Kirson, I., & Lavie, D. (1971). 5, 20α (R)-dihydroxy-6α, 7α-epoxy-1-oxo-(5α) witha-2, 24-dienolide, a new steroidal lactone from *Withania coagulans*. *Phytochemistry*, *10*(3), 685-688.
61. Tursunova RN, Maslennikova VA, Abubakirov NK (1977). Withanolides in the vegetable kingdom. Chem. Nat. Comp. 13:131-138.
62. Velde VV, Lavie D, Budhiraja RD, Sudhir S, Garg KN (1983). Potential biogenetic precursors of withanolides from *Withania coagulans*. *Phytochemistry* 22:2253-2257.
63. Verma P, Srivastava SK, Nath A (2009). Stimulatory effect of *Withenia somnifera* on secretion and endocytotic pathways in the hepatic cell of *Clarias batrachus* (Linn.). *J. Ecophysiol. Occupat. Health* 9:203-209.
64. Watt GA (1972) Dictionary of the economic Products of India. Cosmo Publication, Delhi, India. 6,309.
65. Xu Y-m, Gao S, Bunting DP, Gunatilaka AAL (2011). Unusual withanolides from aeroponically grown *Withania somnifera*. *Phytochemistry* 72:518-522.

PHARMACEUTICAL COCRYSTALS

P. A. Suchetan
Department of Chemistry,
University College of Science, Tumkur University, Karnataka 572103, India
Corresponding author E-mail: pasuchetan@gmail.com

Abstract:

Pharmaceutical cocrystals are recently attracting much attention in the pharmaceutical industry as their physicochemical properties may be modulated in a systematic and predictable manner. In view of its growing importance and successful release of pharmaceutical cocrystals as therapeutic drugs in market, a brief overview on pharmaceutical cocrystals has been presented here. Significance of cocrystals-especially pharmaceutical cocrystals, criteria for coformer selection, physicochemical properties of cocrystals, various techniques of preparation and characterization of cocrystals, and some commercially available cocrystal drugs are discussed in the article.

Introduction:

Drugs are classified, based on their solubility and permeability characteristics, into four major categories as in Figure 1.1 [Duarte *et al.* (2017)]. This classification is called as Biopharmaceutics Classification System (BCS) of drugs. Drugs belonging to Class II and Class IV face problem of poor aqueous solubility which result in poor absorption, low bioavailability and therefore poses challenges during drug development process. Hence, it is imperative to enhance bioavailability of poorly water-soluble class II and class IV drugs in order to improve their drug efficiency.

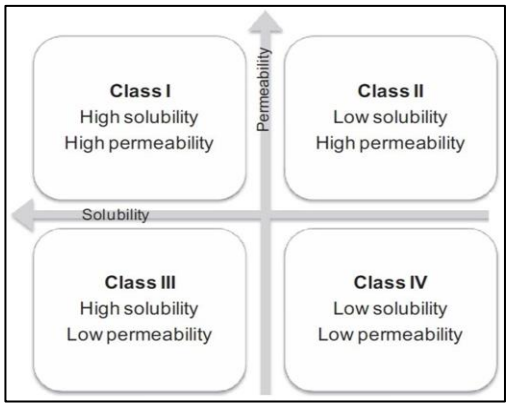


Figure 1.1: Schematic representation of BCS of Drugs [Duarte *et al.* (2017)].

Enhancing aqueous solubility of poorly water-soluble drugs and thereby increasing their bioavailability and drug efficiency is one of the major challenges ahead of the pharmaceutical industries during drug discovery and development process [Kawabata *et al.* (2011)]. The major issue or obstacle faced by the industries is enhancing the efficiency of the drug with no compromise on their chemical nature and stability [Kawabata *et al.* (2011)].

Some of the methods that are employed by the pharmaceutical industries to improve the drug solubility are outlined in the Figure 1.2 [Patel *et al.* (2012)]. Among these, crystal

engineering is considered to be one of the promising techniques, in which the vast library/database of knowledge on supramolecular aggregation or recognition in solid state is made used to modify the API in order to improve its physico-chemical properties [Patel *et al.* (2012)]. Crystal Engineering can be used to tailor the physicochemical properties of APIs [Ter Horst *et al.* (2009)] such as melting point, dissolution rate, aqueous solubility, refractive index, surface activity, habit, density, electrostatic, mechanical and optical properties.

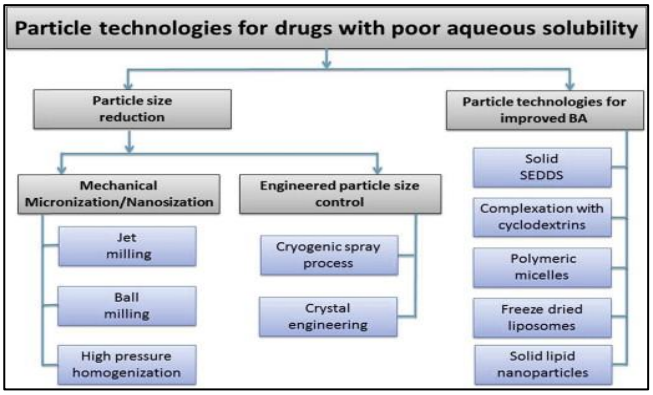


Figure 1.2: A summary of different methods available for modifying aqueous solubility of drugs [Patel *et al.* (2012)].

The advantage of this method is that it avoids the need to start the API development from scratch and thereby avoids the risk of dealing with a poorly water-soluble drug in formulation development stages. Two of the established methods in crystal engineering domain to improve the aqueous solubility of any existing API is to either strategically modify them into their salt form or cocrystallize them with an appropriate coformer. The latter technique is called cocrystallization and the obtained solid is referred to as a cocrystal. As most of the APIs are acidic or basic molecules, one of the appropriate ways to improve the aqueous solubility and dissolution rate of an API is to modify them into their salt forms-as salts of acidic and basic drugs have, in general, higher aqueous solubilities than their native forms. Alkali metal salts of acidic drugs like penicillin and strong acid salts of basic drugs like atropine are more water soluble than their parent drugs- are few examples justifying the utility of the above strategy. The formulation of salt forms of APIs, however, has a serious drawback as it transforms the chemical nature of the APIs and thereby may vary its drug behaviour or action. Therefore, it is critical to improve the solubility of APIs without altering or compromising its chemical nature. One such method for formulation is via cocrystallization.

Cocrystallization is one of the crystal engineering approaches employed to design multicomponent pharmaceutical crystals to improve the dissolution behaviours of otherwise poorly water-soluble APIs without affecting their intrinsic properties [Schultheiss N. and Newman A. (2009)]. Cocrystals are defined as “crystalline materials comprised of two or more components that are solids under ambient conditions and held together by non-covalent forces”. Pharmaceutical cocrystals i.e., the multicomponent cocrystals in which at least one component is an API (at least one component of the crystal lattice is an API) have recently gained much attention in the pharmaceutical industry. The cocrystals offer the advantage of improving the physiochemical properties of the APIs without altering their structural integrity, as the cocrystal components are held together by intermolecular interactions. Hence the formulation of new solid

forms of APIs via cocrystallization method to improve physiochemical properties like melting point, stability, solubility and hygroscopicity without disturbing their chemical properties has gained a wider scope and interest. Especially, design of cocrystals have gained a tremendous momentum in the pharmaceutical industries because of their ability to fine-tune the physicochemical properties of drugs by judicious and knowledge-driven selection of coformers. Regulatory agencies such as United States Food and Drug Administration (USFDA) and European Medicine Agency (EMA) has provided distinct definition for these pharmaceutical cocrystals. According to USFDA, cocrystals are "crystalline materials composed of two or more molecules in the same crystal lattice" or "multi-component solid crystalline supramolecular complexes composed of two or more components within the same crystal lattice wherein the components are in a neutral state and interact via non-ionic interactions" [USFDA (2013)]. EMA defines cocrystals as "homogenous (single phase) crystalline structures made up of two or more components in a definite stoichiometric ratio where the arrangement in the crystal lattice is not based on ionic bonds (as with salts)". The term cocrystal was first used in 1963 by Lawton and Lopez in their patent that discussed cocrystals involving bisphenol and organic amines.

A rational approach in selection of two components of a cocrystal (or selection of a coformer in pharmaceutical cocrystal) such as complementarity between functional groups leading to predictable supramolecular recognition via intermolecular interactions, structural compatibility and stoichiometry regulate successful formation and structure of cocrystal during a cocrystallization event [M. Singh *et al.* (2023)]. Friedrich Wohler, in the year 1844, synthesized the first ever known cocrystal 'quinhydrone' by using benzoquinone and hydroquinone [Wöhler *et al.* (1844)]. It was the first cocrystal structure reported in Cambridge Structural Database (CSD). Subsequently, several API co-crystals were reported well before the formal beginning of the directed synthesis of pharmaceutical co-crystals [Bevill *et al.* (2014)]. Thereafter, there has been a significant leap in the number of cocrystal structures deposited in the CSD signifying its importance in designing promising and marketable drug candidates. Therefore, in this context, aspects such as significance of pharmaceutical cocrysals, criteria for coformer selection, physicochemical properties of cocrystals, various techniques of preparation and characterization of cocrystals, and some commercially available cocrystal drugs are discussed in the article.

Designing Cocrystals: Choice of coformer

1. Solubility and safety

Cocrystals can be divided into two major groups, namely molecular cocrystals and ionic cocrystals. Molecular cocrystals contain two or more different neutral components and are sustained by hydrogen bonds or halogen bonds. In contrast, ionic cocrystals contain at least one ionic component and are supported by charge-assisted hydrogen bonds or coordination bonds if metal cations are present. The coformer, typically an organic molecule, are generally chosen on rational grounds. First and foremost, coformers should have better aqueous solubility and should be less dangerous or less toxic. Such coformers are termed as 'Generally Regarded As Safe (GRAS)' chemicals by USFDA. For instance, amino acids are soluble and stable in water, and are also regarded as GRAS chemical, thus facilitating the "green method" cocrystallization.

2. Complementary functional groups and hydrogen bonding

Coformers should have complementary hydrogen bonding sites that can interact with the API's functional groups in terms of non-covalent interactions such as hydrogen bonds, intermolecular interactions, halogen bonds etc. As an example, the amino and carboxylic groups of amino acids can serve as hydrogen bond donor and acceptor groups, and thereby form hydrogen bonds with other groups, such as hydroxyl, carboxyl, pyridyl, phenolichydroxyl and several others [Cherukuvada, S. and Guru Row, T.N. (2014)]. Functional groups that can form supramolecular synthons are vital to form intermolecular hydrogen bonds during cocrystal synthesis. Additionally, molecules with multiple functionalities that can form multiple hydrogen bonds are more likely to form cocrystals and that too with different stoichiometry. In a cocrystal, the nature of hydrogen bonds are being generally dictated by the hydrogen bond rule which states that 'best hydrogen bond donor tends to interact with the best hydrogen bond acceptor in a given crystal structure', thereby ensuring a robust and directional nature to it. This 'best-donor–best-acceptor' rule can be of great use in designing cocrystals with specific hydrogen bonding interactions.

The API molecules contain certain functional group (or molecular recognition point) in their structure which interacts with the coformer and thereby create a supramolecular unit (or molecular recognition point) called supramolecular synthons [Almarsson Ö. and Zaworotko M. J. (2004)]. The term 'Synthon' was first introduced in 1967 and is defined as *"structural units within supermolecules which can be formed and/or assembled by known or conceivable synthetic operations involving intermolecular interactions"* [Corey, E. J. (1967)]. Supramolecular synthons are also defined as "spatial arrangement of intermolecular interactions which serves as a base for any supramolecular synthesis" [Desiraju, G.R. (1995)]. Thus, synthons are design elements in crystal engineering and should not be confused with the term 'intermolecular interactions' [Desiraju, G.R. (1995)]. The supramolecular synthons are classified as homosynthons and heterosynthons depending on the nature of the complementary functional groups in the API and coformer molecules via which they are interacting with each other. Homosynthons are formed due the interaction between identical self-complementary functional groups such as acid…acid and amide…amide groups (Figure 1.3) whereas the heterosynthon formation arises due to the interaction between two different functional groups (such as acid…amide, acid…pyridine and amide…pyridine groups (Figure 1.4). Heterosynthons could also be formed as a result of halogen bonding.

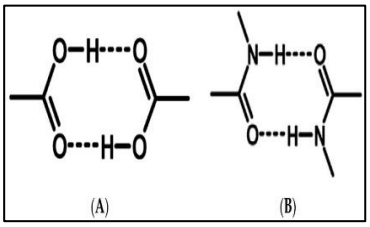


Figure 1.3: Schematic representation of supramolecular homosynthons: (A) acid-acid dimer and (B) amide-amide dimer.

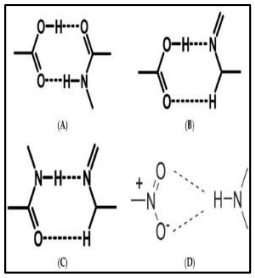


Figure 1.4: Schematic representation of supramolecular heterosynthons: (A) acid-amide dimer, (B) acid-pyridine dimer, (C) amide-pyridine dimer and (D) Nitro-amine interaction [Berry D. J. and Steed J. W. (2017)].

3. ΔpKa rule

ΔpKa value has been used to assess cocrystal formation ability of a coformer with a given API [Berry D. J. and Steed J. W. (2017)]. pKa value indicates the ability of an acid molecule to give up a proton. When the difference between pKa value of API and coformer (ΔpKa) ranges in negative values, there will be no proton transfer. Therefore, one can possibly expect cocrystal formation in such a case [Berry D. J. and Steed J. W. (2017)]. On the other hand, salt formation is observed when ΔpKa value is greater than 2 or 3 due to completion of proton transfer [M. Singh *et al.* (2023) ;Childs, S. L. *et al.* (2007)]. However, when ΔpKa value is somewhere between 0 and 1, the outcome cannot be predicted accurately as both cocrystallization and salt formation are reported in literature in this range.

Types of solid forms obtained from cocrystallization

While cocrystal is the ultimate desired product, an unsuccessful cocrystallization event might end up in formation of different solid forms such as salts, solvates, hydrates, eutectics [Goud N. R. *et al.* (2012)], a drug polymorph [Indumathi S. and Sameer V. D. (2017)], coamorphous solids, physical mixtures, and solid solutions. These solid forms can contain of a single component or multiple components. Single component solids constitute polymorphs and amorphous forms. Multicomponent solids constitute cocrystals, polymorphs of cocrystals, salts, solvates/hydrates, coamorphous solids, and continuous solid solutions or discontinuous solid solutions/eutectics. Formation of different solid forms of an API are depicted in Figure 1.5 [Goud N. R. *et al.* (2012)].

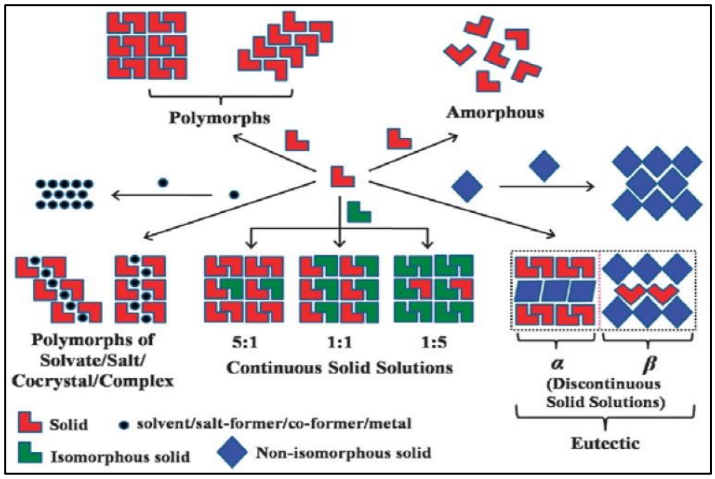


Figure 1.5: Different solid forms [Goud N. R. *et al.* (2012)].

Difference between cocrystals, salt, solvates and hydrate:

Polymorphs are the compounds which are present in different crystalline forms such as solvates or hydrates and amorphous forms. They have different arrangement of lattice (i.e., crystal lattice structures) and so they have different physicochemical properties. Cocrystals and solvates can be differentiated based on their physical state of the components. Presence of a liquid component constitutes a solvate whereas those which contain only solid components are cocrystals [David J. and Nair R. H. (2009)]. If the solvates contain water in their crystal lattice, then they are known as hydrates. Solvates/hydrates are common during the cocrystallization-especially when cocrystals are prepared from solution evaporation or liquid assisted grinding methods. The solvated/hydrated forms can alter physicochemical properties of API's. They are relatively unstable as they lose solvent at high temperatures and low humidity during storage. Hence, the only difference between a solvate and a cocrystal is the physical state of the components [Shilpa C.N. *et al.* (2018)]. Figure 1.6 demonstrates the formation of a cocrystal and its solvate/hydrate [David J. and Nair R. H. (2009)].

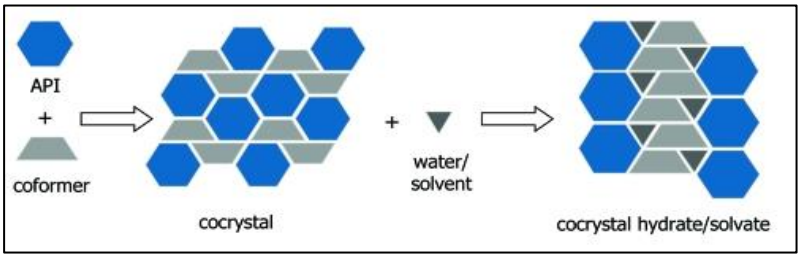


Figure 1.6: Formation of a cocrystal and its hydrate/solvate [David J. and Nair R. H. (2009)].

Cocrystal versus Salt Formation: Salts are formed by complete transfer of a proton from one compound to another. Salts and cocrystals can be differentiated based on whether a proton transfer has taken place or not. A complete proton transfer takes during salt formation, whereas, no such event occurs during cocrystallization. Formation of salt or a cocrystal can be predicted using the ΔpKa rule as stated earlier. Salt formation and cocrystallization are two different processes, though both are used for enhancing solubility, stability, and other aspects of the API formulation. While salt formation needs an API charge to form its salt form, cocrystallization doesn't need any such criteria. Hence, cocrystallization offers an option for API which doesn't carry a charge and needs to be enhanced for solubility or stability [David J. and Nair R. H. (2009)].

Properties of cocrystals

Cocrystallization involves alteration of physical properties of an API by modifying the drug at molecular level. It is therefore possible to improve certain physicochemical properties of a drug by a judicious selection of the coformer. Some of the properties that could be altered by cocrystallization are summarized in this section. The modification of physicochemical properties (without compromising the pharmacological properties of API) largely depends on the individual properties of both API and the coformer and nature of interaction between the two. The enhancement of different physicochemical properties of drugs are discussed here with suitable examples.

***Melting point*:** Melting point is an important physical property for characterizing and identifying the purity of cocrystals. Melting points of cocrystals are generally obtained from Differential

Scanning Calorimetric (DSC) technique. Cocrystals having melting points lower than their API are usually preferred as they would show lower susceptibility towards degradation. DSC method is the preferred over a standard melting point apparatus, as additional thermal data such as the enthalpy of fusion, different phase transitions etc can be determined from DSC profile. These additional data become essential especially when attempting to characterize a polymorphic pair of compounds as monotropic or enantiotropic [Shilpa *et al.* (2018), Edislavleksic and Gordanapavlovic (2012)].

***Tabletability*:** Tabletability means ability of a substance to get converted into a tablet form. Crystal packing, tabletability and compaction are important parameters of preformulation study. APIs with potential to form marketable drugs fail to reach markets due to their poor ability to get converted into a tablet form. However, it is possible to alter these properties of API with the help of cocrystallization technique by using suitable conformers. For instance, cocrystals of Resveratrol with coformers 4-aminobenzamide and isoniazid has shown enhanced solubility and tabletability [Huang L.-F. and Tong W.-Q. (2004)]. Cocrystals showed higher tabletability than either pure drug or coformers. Similarly, compaction behaviour of cocrystals of paracetamol with trimethyl glycine and oxalic acid was found to be better than pure drug [Huang L.-F. and Tong W.-Q. (2004)]. The mechanical properties of APIs could also be altered by varying crystal packing via cocrystallization. For example, paracetamol have poor compression property, and to overcome this limitation, wet granulation method is generally employed for tablet preparation. As this method itself is very tedious, to overcome this difficulty, cocrystals of paracetamol with caffeine were prepared which significantly enhanced its mechanical property such as compaction and tabletability [Huang L.-F. and Tong W.-Q. (2004)].

***Solubility*:** Cocrystals have enhanced solubility which increases the rate of dissolution-the rate limiting step in gastrointestinal absorption. The limitations of traditional methods of solubility enhancement such as configuration of salt, solid dispersion and micronization, etc. are overcome by cocrystals via thermodynamic and kinetic approaches [Huang L.-F. and Tong W.-Q. (2004)]. For example, cocrystal of ketoconazole and ascorbic acid prepared from slurry method showed an improved solubility by 50 times compared to ketoconazole [Budziak-Wieczorek I. and Maciołek U. (2021)].

Stability: Stability is an important parameter for the design of a dosage form. During cocrystallization there is modification in molecular assemblies that alters the mechanical properties of solids. Hence, study of stability of polymorphic cocrystal is important. There are four aspects related to the stability of a pharmaceutical cocrystal. They are relative moisture stress, heat stress, and chemical & solution stability. The product's best storage state is assessed by the presence of relative moisture, as water in the cocrystals causes the loss of its quality. In the case of cocrystals and salts, solution stability may be a factor due to dissociation of the material resulting in precipitation of the less soluble parent compound or a less soluble form (such as a hydrate in aqueous media). For example, polymorphic cocrystals of carbamazepine with coformers saccharin and nicotinamide are more stable than the original API. Other parameters such as tensile strength, elastic properties, breaking strength and tabletability also significantly improved [Huang L.-F. and Tong W.-Q. (2004)].

***Bioavailability*:** Bioavailability is defined as the rate and extent of pure drug that reaches into systemic circulation. Low oral bioavailability of APIs is one of the major challenges in development and formulations of drugs. Increasing the drug's solubility increases the

bioavailable fraction of the drug that reaches systemic circulation, and thereby, indirectly enhances the therapeutic efficacy of any API. Cocrystallization is an efficient method which enhances the solubility the bioavailability of a drug. Several research have been conducted to enhance the bioavailability of different drugs with conversion into cocrystal form [Budziak-Wieczorek I. and Maciołek U. (2021)]. The example of Meloxicam cocrystals with aspirin exhibited better oral bioavailability as compared to pure drug and showed 12 times faster onset of action than pure drug in rats [Srivastava D. *et al.* (2018)].

Different techniques for the preparation of cocrystals

Different techniques for the preparation of cocrystals are summarized in this section.

1. Solvent evaporation technique:

This is the most commonly used technique in the field of cocrystallization. In this technique, stoichiometric amount of the coformer and the API are dissolved in a common solvent. It is based on the principle that, 'when different molecules of complementary functional groups afford hydrogen bonds that are more favourable than each of the individual molecular components, the product formed is likely to be thermodynamically favoured' [Karimijafari, M. *et al.* (2018); Sheetal, B. and Kevin C. G. (2020)]. This technique is also useful for obtaining good quality single crystals suitable for single crystal X-ray diffraction studies (SCXRD).

2. Solid-state grinding technique (including solvent drop technique):

This technique is also known as mechanical milling or neat grinding technique. In this method, the two cocrystal formers are taken in stoichiometric amounts and ground together using a mortar and pestle or using a ball mill or using a vibratory mill. Grinding time generally ranges from 30-60 min. This method yields powder form of cocrystal, and not a single crystal. Therefore, this method unlike solvent evaporation method does not afford single crystals required for SCXRD studies. In such cases, cocrystals can be first produced by this technique which may later be used as seeds to obtain good quality single crystals of cocrystal by another method, thus facilitating XRD structure determination via single-crystal growth [Burande, S. *et al.* (2021)].

A recent modification to the solid-state grinding method is to add small amounts of solvent during the grinding process. This method is called liquid assistant grinding method (LAG) or kneading [Karimijafari, M. *et al.* (2018); Anuja, S. *et al.* (2020)]. LAG method enhances the kinetics and facilitates cocrystal formation, and is a preferred method of preparing cocrystals over solid-state grinding method. This method has other advantages over solid state grinding, such as increased yield, control over polymorph formation, better crystallinity of the cocrystal etc.

3. Slurrying technique:

Slurry-induced formation of cocrystalline phase is among two or more active solid materials or between the active solid materials and the excipients. Equimolar proportion of the two coformers are dissolved in small amounts of different solvents at ambient temperature and allowed to stir for some days. The resulting solution is slowly evaporated at room temperature during 48 hours to promote cocrystallization. Further, the solvent is allowed to decant and the solid material is dried under a flow of nitrogen for 5 min. The remaining solids can be characterized using powder X-ray diffraction (PXRD) [Chaudhari, S. *et al.* (2018); Anuja, S. *et al.* (2020)].

4. Use of intermediate phase:

Cocrystals can also be formed by using intermediate phases. In the course of time the use of a hydrate or an amorphous phase as an intermediate during synthesis in a solid-state route has proven successful in forming a cocrystal. Besides this, the use of a metastable polymorphic form of one cocrystal former can be used. The metastable form in this technique acts as an unstable intermediate on the nucleation pathway to a cocrystal [Chaudhari, S. *et al.* (2018); Anuja, S. *et al.* (2020)].

5. Antisolvent addition:

Antisolvent addition also known as vapor diffusion is the methods in which antisolvent is used to synthesize cocrystals. This is one of the methods for precipitation or recrystallization of the two cocrystal former. Solvents consist of buffers (pH) and organic solvents. For example, in the preparation of cocrystals of aceclofenac using chitosan, the chitosan solution was prepared by soaking chitosan in glacial acetic acid. A stoichiometric amount of the drug was suspended in chitosan solution by using high dispersion homogenizer. Further, the dispersion was added to distilled water or sodium citrate solution to precipitate chitosan on drug [Karimijafari, M. *et al.* (2018)].

Characterization of cocrystals

The different instrumental analytical techniques used in the characterization of the cocrystals are discussed here.

1. X-ray diffraction (XRD) studies - Single crystalline and powder XRD.

Diffraction is an analytical tool employed for phase identification of unit cells associated with the cocrystal. Complete structural information of cocrystals can be obtained from single and powder X-ray crystallography. Powder XRD (PXRD) is regularly used in the identification of various cocrystals by detecting changes in the crystal lattice since different characteristic peaks are associated with different cocrystals, while single crystal XRD is mainly employed for structural recognition and complete structural information [Burande, S. *et al.* (2021)]. Difficulty in the procurement of a single crystal is the main problem associated with single crystal XRD. Formation of cocrystals is confirmed by combining different tools such as PXRD, differential scanning calorimetry (DSC), FT-IR etc when quality single crystals for SCXRD studies are not available. The utility of diffraction technique is summarized using two examples. 1) Comparison of PXRD spectra of Betulinic Acid (BA), Vitamin C (VitC), and BA-VitC cocrystal shows that the spectrum of the cocrystal exhibit significant differences as compared to the ones recorded for the pure compounds, thus suggesting the formation of the cocrystal. The PXRD spectrum of the physical mixture was different from that of the cocrystal, and was just the sum of the diffraction peaks recorded for the pure compounds (BA and VitC) lacking any new diffraction pattern [Nicolov, M. *et al.* (2019)]. Therefore, the presence of interactions between the coformers in the cocrystal can be validated by analysing the PXRD spectrum. 2) Cocrystal of Epicatechin (EC) with Barbituric Acid (BTA) was characterised by single crystal diffraction data. These compounds display structural similarities, as well as significantly distinct crystallization behaviour.

2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) is widely used for the characterization of cocrystals in the pharmaceutical field. In this technique, the cocrystal and pure components are heated at a controlled rate and the obtained thermogram is scrutinized

for checking the possibility of cocrystal formation. In this process, eutectic melt generated at slow heating rates recrystallizes to the cocrystal form and then melts, irrespective of the ratio of drug and coformer. The thermogram obtained by the DSC scan is used for screening of cocrystals as it allows the cocrystal detection. The thermogram of cocrystals shows a different exothermic peak from that of the pure thermogram of drug and conformer followed by an endothermic peak. The melting point and heat of fusion detected for cocrystals will be different from that of the pure components. If a physical mixture that cannot form cocrystals is heated, then only a single endothermic peak associated with eutectic melting can be seen in thermogram [Sheetal, B. and Kevin C. G. (2020)]. For example, characteristic and comparable thermograms of Betulinic Acid (BA), Vitamin C (VitC) and their cocrystal have been studied [Nicolov, M. *et al.* (2019)]. The DSC curve of BA+VitC cocrystal shows significant differences when compared with those of BA and VitC, thereby suggesting the cocrystal formation.

3. Spectroscopic techniques

In vibrational spectroscopy (infrared and Raman), the energy absorbed or scattered by the chemical bonds in the cocrystals will be different from that of the pure components. This leads to the identification of the structural behaviour of cocrystals. In IR spectroscopy, cocrystals show a different spectrum of bands from the pure drug and coformer due to the formation of hydrogen bonding between them. A clear difference is seen in the bands of functional groups which have undergone hydrogen bonding. For example, a neutral carboxylic acid (COOH) group shows a stronger and weaker intense band of C=O at about 1700 cm^{-1} and 1200 cm^{-1}, respectively, and carboxylic anion (COO^-) shows a weak intense band in between 1000-1400 cm^{-1} due to the formation of salts. If the H-bonding occurs between OH....N, then two broad zones will be witnessed at about 2450 cm^{-1} and 1950 cm^{-1}[Burande, S. *et al.* (2021)].

An example of FTIR spectral studies of Betulinic (BA) acid and Vitamin C (VitC) reveal the binary adduct formation between the components, by the shifting of bands from the crystal to lower or higher wavenumbers, outside the range of ±5 cm^{-1}. At high wavenumbers (spectral range 3,600–2,500 cm^{-1}), the stretching of simple bonds such as O-H and C-H occurred. The broad band observed in the spectral range 3,600–3,400 cm^{-1} reveal the presence of intermolecular bonds, both in the case of pure BA, as in the case of the BA+VitC cocrystal. This observation leads to the conclusion that the formation of cocrystal occured by hydrogen bonding [Nicolov, M. *et al.* (2019)].

Solid-state nuclear magnetic resonance is widely used for characterization of pharmaceutical cocrystals due to their ability to provide structural information of cocrystals. This method is also used to distinguish cocrystals and salts since it can detect the degree of proton transfer. This method is advantageous especially when suitable single crystals are not available for complete structural elucidation by SCXRD studies. One of the main disadvantages of this method is the low sensitivity of the instrument involved in measurement [Burande, S. *et al.* (2021)]. Solid state NMR is advantageous over solution NMR for cocrystals because, in solution NMR, spectra consist of a series of very sharp transitions, due to averaging of anisotropic NMR interactions by rapid random tumbling. By contrast, solid-state NMR spectra are very broad, as the full effects of anisotropic or orientation-dependent interactions are observed in the spectrum.

4. Field emission scanning electron microscopy (FESEM)

FESEM or topography is used to study the surface morphology of cocrystals. Micrographs of components and cocrystals obtained in the FESEM studies are utilized for the

comparison. In the field emission electron microscope, heat energy is not used and so-called "cold" source is employed. A strong electric field is utilized to emit the electrons from the surface of the conductor. A tungsten filament with a thin and sharp needle (tip diameter 10–100 nm) is employed as a cathode. The field emission source is attached with a scanning electron microscope for the capture of micrographs of cocrystals [Burande, S. *et al.* (2021)]. This method, however, is not useful for structural characterization of the cocrystal.

5. Hot Stage Microscopy

A combination of microscopy and thermal analysis is included in the hot stage microscopy study. The physicochemical characteristic of a solid form is studied as a function of temperature and time. The changes occurred while heating the cocrystal sample placed on a glass slide are clearly observed under the microscope for assessing the changes such as melting point, melting range, and crystalline transformation [Karimijafari, M. *et al.* (2018)].

Some commercial cocrystal drugs

Some cocrystal drugs readily available in market are mentioned below to signify their importance in pharma industries. These cocrystal drugs are available in market and are known to have better performance than standalone APIs.

1. Cocrystal of Nifedipine and Isonicotinamide has superior photostability than the API [Wang X. *et al.* (2021)].
2. Cocrystal of Moxifloxacin with trans-cinnamic acid has an improved residence time in the lungs [Wang X. *et al.* (2021)].
3. Cocrystal of Temozolomide and Baicalein has better plasma drug concentration [Wang X. *et al.* (2021)].
4. Cocrystal of Posaconazole and 4-aminobenzoic acid has superior solubility and dissolution behavior than the individual API [Kuminek G. *et al.* (2021)].
5. Cocrystal of Ipragliflozin and L-proline potently and selectively inhibited human and rat at nanomolar ranges and exhibited stability against intestinal glucosidases [Tahara A. *et al.* (2012)].

Conclusion:

The article describes the significance of pharmaceutical cocrystals as potential candidate for therapeutic drugs by addressing issues such as its strategic design by judicious selection of coformer, modulation of its physicochemical properties when compared to original API, and different methods of preparation and characterization of cocrystals.

References:

1. Duarte, A. R. C.; Ferreira, A. S. D.; Barreiros, S.; Cabrita, E.; Reis, R. L.; Paiva, A. (2017), A Comparison between Pure Active Pharmaceutical Ingredients and Therapeutic Deep Eutectic Solvents: Solubility and Permeability Studies. European Journal of Pharmaceutics and Biopharmaceutics, 114, 296–304.
2. Kawabata, Y.; Wada, K.; Nakatani, M.; Yamada, S.; Onoue, S. (2011), Formulation Design for Poorly Water-Soluble Drugs Based on Biopharmaceutics Classification System: Basic Approaches and Practical Applications. International Journal of Pharmaceutics, 420 (1), 1–10.
3. Patel, C. J, Asija, R.; Asija, S. (2012), Different methods of enhancement of solubilization and bioavailability of poorly soluble drugs: A recent review. PharmaTutor.

https://www.pharmatutor.org/ articles/different-methods-enhancement-solubilization-bioavailability-poorly-soluble-drugs-review-1424.

4. Ter Horst, J. H.; Deij, M. A.; Cains, P. W. (2009), Discovering New Co-Crystals. Crystal Growth & Design, 9 (3), 1531–1537.
5. Schultheiss, N.; Newman, A. (2009), Pharmaceutical Cocrystals and Their Physicochemical Properties. Crystal Growth & Design, 9 (6), 2950-2967.
6. US Food and Drug Administration. Guidance for Industry: Regulatory Classification of Pharmaceutical Co-Crystals. Centre for Drug Evaluation and Research; Silver Spring, MD, USA: 2013.
7. M. Singh, H. Barua, V.G. S. S. Jyothi, M. R. Dhondale, A. G. Nambiar, A. K. Agrawal, P. Kumar, N. R. Shastri and Dinesh Kumar (2023), Cocrystals by Design: A Rational Coformer Selection Approach for Tackling the API Problems, *Pharmaceutics*, *15*, 1161.
8. Wöhler, U. das Chinon, J. L. der Chemie (1844) - Wiley Online Library 1844. https://chemistry-europe.onlinelibrary.wiley.com/
9. Bevill, M. J.; Vlahova, P. I.; Smit, J. P. (2014) Polymorphic Cocrystals of Nutraceutical Compound P-Coumaric Acid with Nicotinamide: Characterization, Relative Solid-State Stability, and Conversion to Alternate Stoichiometries. Crystal Growth & Design, 14 (3), 1438-1448.
10. Cherukuvada, S.; Guru Row, T. N. (2014) Comprehending the Formation of Eutectics and Cocrystals in Terms of Design and Their Structural Interrelationships. Crystal Growth & Design, 14 (8), 4187–4198.
11. Almarsson, Ö.; Zaworotko, M. J. (2004), Crystal Engineering of the Composition of Pharmaceutical Phases. Do Pharmaceutical Co-Crystals Represent a New Path to Improved Medicines? Chem. Commun., No. 17, 1889–1896.
12. Corey, E. J. (1967), General Methods for the Construction of Complex Molecules. Pure and Applied Chemistry, 14 (1), 19–38.
13. Desiraju, G.R. (1995), Supramolecular Synthons in Crystal Engineering—A New Organic Synthesis, Angewandte Chemie, 34 (21), 2311–2327.
14. Berry, D. J.; Steed, J. W. (2017), Pharmaceutical Cocrystals, Salts and Multicomponent Systems; Intermolecular Interactions and Property Based Design. Advanced Drug Delivery Reviews, 117, 3–24.
15. Childs, S. L.; Stahly, G. P.; Park, A. (2007), The Salt−Cocrystal Continuum: The Influence of Crystal Structure on Ionization State. Mol. Pharmaceutics 2007, 4 (3), 323–338.
16. Goud, N. R.; Suresh, K.; Sanphui, P.; Nangia, A. (2012), Fast Dissolving Eutectic Compositions of Curcumin. International Journal of Pharmaceutics, 439 (1), 63–72.
17. Indumathi, S.; Sameer, V. D. (2017), Co-amorphous Form of Curcumin–Folic Acid Dihydrate with Increased Dissolution Rate, Crystal Growth & Design, 17 (12), 6273–6280.
18. David, J.; Nair, R.-H. (2009), Solubility Advantage of Pharmaceutical Cocrystals, Crystal Growth & Design, 9 (5), 2252–2264.
19. Shilpa, C. N.; Sarika, K. Neetu, W. Shubham; D. Y. Patil, (2018), Co-Crystals: A Review, Journal of Drug Delivery & Therapeutics, 8 (6), 350–358.

20. Edislavleksic.; Gordanapavlovic. (2012), Cocrystals of Lamotrigine Based on Coformers Involving Carbonyl Group Discovered by Hot-Stage Microscopy and DSC Screening, Crystal Growth & Design, 12 (4), 1847–1858.
21. Huang, L.-F.; Tong, W.-Q. (Tony) (2004). Impact of Solid-State Properties on Developability Assessment of Drug Candidates. Advanced Drug Delivery Reviews, 56 (3), 321–334.
22. Budziak-Wieczorek, I.; Maciołek, U. (2021), Synthesis and Characterization of a (−)-Epicatechin and Barbituric Acid Cocrystal: Single-Crystal X-Ray Diffraction and Vibrational Studies. ACS Omega, 8199–8209.
23. Srivastava, D.; Fatima, Z.; Kaur, C. D. (2018), Multicomponent Pharmaceutical Cocrystals: A Novel Approach for Combination Therapy. Mini-Reviews in Medicinal Chemistry 18 (14), 1160–1167.
24. Karimijafari, M.; Padrela, L.; Walker, G.; Croker, D. (2018), Creating Cocrystals: A Review of Pharmaceutical Cocrystal Preparation Routes and Applications. Crystal Growth & Design, 18 (10), 6370–6387.
25. Sheetal, B.; Kevin C. G. (2020), Pharmaceutical Cocrystals-A Review. MDPI, Proceedings 2020, 62, 14; doi:10.3390/proceedings2020062014.
26. Burande, S.; Gandhi, P.; Chakole, R.; Charde, M.; Burade, K. (2021), Preparation and Characterization of Co-Crystals: A Review. International Journal of All Research Education & Scientific Methods, 9, 1755–1763.
27. Anuja, S.; Virsh, K.C..; Abdul, R.T.;, Shabaraya, A. R. (2020), Pharmaceutical co-crystals: An overview; World Journal of Pharmacy and Pharmaceutical Sciences, 9(11), 556-569.
28. Chaudhari, S.; Nikam, S.; Khatri, N.; Wakde, S. (2018), Co-crystal: A review. Journal of Drug Delivery and Therapeutics, 8, 350–358.
29. Nicolov, M.; Ghiulai, R. M.; Voicu, M.; Mioc, M.; Duse, A. O.; Roman, R.; Ambrus, R.; Zupko, I.; Moaca, E. A.; Coricovac, D. E.; Farcas, C.; Racoviceanu, R. M.; Danciu, C.; Dehelean, C.-A.; Soica, C. (2019), Cocrystal Formation of Betulinic Acid and Ascorbic Acid: Synthesis, Physico-Chemical Assessment, Antioxidant, and Antiproliferative Activity. FrontChem, *7*, 92.
30. Wang, X.; Du, S.; Zhang, R.; Jia, X.; Yang, T.; Zhang, X. (2021), Drug-Drug Cocrystals: Opportunities and Challenges. Asian Journal of Pharmaceutical Sciences, 16 (3), 307–317.
31. Kuminek, G.; Cavanagh, K. L.; da Piedade, M. F. M.; Rodríguez-Hornedo, N. (2019), Posaconazole Cocrystal with Superior Solubility and Dissolution Behavior. Crystal Growth & Design, *19* (11), 6592–6602.
32. Tahara, A.; Kurosaki, E.; Yokono, M.; Yamajuku, D.; Kihara, R.; Hayashizaki, Y.; Takasu, T.; Imamura, M.; Qun, L.; Tomiyama, H.; Kobayashi, Y.; Noda, A.; Sasamata, M.; Shibasaki, M. (2012), Pharmacological Profile of Ipragliflozin (ASP1941), a Novel Selective SGLT2 Inhibitor, in Vitro and in Vivo. Naunyn-Schmiedeberg's Arch Pharmacol, *385* (4), 423–436.

INHIBITORY EFFICACY OF PLANT THERAPEUTIC COMPOUNDS AGAINST PATHOGENIC MICROBES

Swapan Kumar Chowdhury
Department of Botany, Balurghat College,
Balurghat, Dakshin Dinajpur, West Bengal, PIN-733101, India,
*Corresponding author E-mail: chowdhuryswapankr3@gmail.com

Abstract:

Emergence of new multidrug resistance (MDR) microbes poses a threat to human and animal communities. The reason for such critical issues is due to unscientific-rampant uses in diseased persons and disposal of potent antibiotics in natural sites, resulting in unwanted exposure of soil microbes to such compounds. As a consequence of such exposure, the list of such emerging MDR is growing day by day, with frequent contamination and infection in the animal life forms. Therefore, the pharmaceutical companies are in utter needs to develop new and novel potential antimicrobials to combat such MDR-diseases. However, our knowledge and skills are still limiting to design and develop such more potential drug molecules for such challenges. Here, the metabolic diversities and biosynthesis machinery of the plant communities, more specifically the medicinal plant species, provide a big canvas to search for that. So, the present pharmaceutical companies are greatly relying on the knowledge of natural lead antimicrobial molecules effective against the MDR strains to be exploited for large scale application in both human and animal life forms. In the present research communication, we are attempting to decipher the biochemistry, efficacy and prospects of such potential lead molecules in the aid of MDR strains.

Keywords: Antimicrobials; Medicinal plants; Multidrug-resistant pathogens and diseases; Primary metabolites; Secondary metabolites

Introduction:

Nature is the source of various medicinal herbs and human depends on it for treatment of ailments and diseases over centuries as they are beneficial for their minimum side effects properties. World Health Organization (WHO) has reported that the 80% of world population mainly the ethnic communities depend on the plant based traditional medicine to fulfill their primary healthcare needs (Nitha *et al.* 2012; Mishra *et al.* 2013). Phytochemicals, mainly the secondary metabolites of plants are used in synthetic drugs of modern medicines worldwide. These are structurally diverse bioactive compounds derived from medicinal plants (Kumar and Pandey, 2013). They have also been used as antimicrobial agents against pathogens to combat infectious diseases (Mishra *et al.* 2013; Chandra *et al.* 2018). Microorganisms such as bacteria, fungi and viruses are the major causal agents of various infectious diseases in humans. The more attention has been cocentrated on the study on plant derived compounds over the past decades which specially act against multidrug-resistant (MDR) Gram-negative and Gram-positive bacteria (Borges et al.,2015). Since the microbial sources are widely relied upon, the plant originated pharmaceutical products distributed about 50% in which few are used as antimicrobials in United States (Subramani *et al.* 2017). The golden era of the discovery of novel

antibiotics was considered from 1950s to 1970s (Aminov, 2010). The term of antimicrobial resistance (AMR) is often used to define the growth and spread of bacteria, fungi, viruses, protozoa, and nematodes in the presence of antibiotics or antimicrobial agents that was originally found to be effective for the treatment of infection caused by that microbes (Founou *et al.* 2017). The higher health care costs, increased duration of illness, and frequency of hospitalization enhanced due to uses of antibiotic resistance pathogens when compared with non-resistant common infections. It has been scientifically proven that the indiscriminate and inappropriate use of antibiotics has accelerated the emergence of multidrug-resistant strains (ELChakhtoura *et al.* 2018). Bacteria are the common microbes in which antibiotic resistance develops either through mutation of a target site protein, through the incorporation of an antibiotic-resistance gene (ARG) that confers resistance through efflux or inactivation of the antibiotic, or through synthesis of a new target protein that is insensitive to the antibiotic (Davies *et al.* 2010). The mortality rate for patients with infections caused by non-resistant bacteria is less than half of that of people with a resistant form of the same infection (Tiwari *et al.* 2013). The few bacterial pathogens have been recognized as most terrible pathogens includingβ-lactamase-producers *Escherichia coli* and *Klebsiella pneumoniae*, carbapenem-resistant Enterobacteriaceae and *Pseudomonas aeruginosa*, hospital acquired methicillin resistant Staphylococcus aureus, and vancomycin resistant Enterococcus (Talbot *et al.* 2006, Kumar *et al.* 2013; Satlin, *et al*, 2017). The enteric pathogens *Pseudomonas aeruginosa*, Streptococcus pneumonia, S. aureus and Mycobacterium tuberculosis amidst modified as multidrug resistance pathogens by the emergence of resistance to multiple antibiotics (Lowy *et al.* 2003, Ballal *et al.* 2014). On the other hands, *Enterococcus faecium*, *S. aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp, are high level of drug resistance bacteria together referred by the acronym ESKAPE which cause the majority of infections within the hospital environment (Pendleton *et al.* 2013). The broad range of AMR mechanisms used by the ESKAPE pathogens includes enzymatic inactivation, modification of drug targets, changing cell permeability through porin loss or increase the mechanical protection and expression of efflux pumps provided by biofilm formation. AMR in these pathogens is a major concern to public health systems worldwide and is likely to increase as resistance profiles change. Antibiotics have been saving an immense number of lives from many infectious diseases from more than a half-century. But due to increase in the AMR the demand for alternative therapeutic compounds against those MDR pathogens has been emphasized for the last few decades. To overcome antibiotic resistance by developing more powerful antibiotics, which has been attempted by pharmaceutical companies, can lead to an only limited and temporary success and eventually contribute to developing greater resistance. There is urgent need to develop some new medicinal plant-derived natural compounds which play an important role as antimicrobial agents with diverse chemical structures and novel mechanisms of action. Researchers and Scientists from different research backgrounds have found thousands of phytochemicals with their antimicrobial usefulness on all types of microorganisms (Cowan 1999). The present study is a review focused on plant derived antimicrobial compounds and their potential applications as novel antimicrobial agents against MDR pathogens and their mechanism of actions.

Plant Secondary metabolites as bioactive molecules

Most of higher plants develop secondary metabolites, which are pharmacologically active. The development of secondary metabolites can either be a part of the plant's normal program of growth, or it can happen in response to pathogenic attack (such as phytoalexins). The secondary metabolites in plants can be divided into different categories according to their biosynthetic principles. A simple classification includes 3 main groups: Terpenes such as mono-, di-, tri-, sesqui- and tetraterpenes, saponins, steroids, cardiac glycosides and sterols; Phenolics such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignins; and Nitrogen containing compounds such as alkaloids and glucosinolates. The compounds within the groups do also have some similarities in their structures (Agostini-Costa, 2012).

Historical perspectives of the use of medicinal plants as antimicrobials

Medicinal plants have provided the source of various novel drug compounds since antiquity which have largely contributed to human health and well-being. Many plants derived phytochemicals used for the natural development of new drugs as well as phytomedicine to be employed for the treatment of infectious diseases. According to Hippocrates in the late century B.C., 300 to 400 plants having medicinal properties (Schultes 1978). The quinine alkaloid obtained from Cinchona sp. has a long history of therapeutic use in the treatment of malaria, relieve nocturnal leg cramps and Staphylococcus aureus (Kushwah P. *et al.,* 2016). The Cephaelis ipecacuanha (Brot.) A. Rich. (Family: Rubiaceae) spread of Escherichia histolytica infections.

Emerging infectious diseases in human: Causes and consequences

Pathogenic microorganisms are known to cause various types of infection in human being leading to death worldwide. A brief overview of some highly disease causing pathogenic microbes have been enumerated below.

1. Bacterial diseases

Among the Bacterial pathogens, Enterobacteriaceae members are responsible for causing several infections of humans (Fabio *et al.* 2007). Several hospital- acquired infections such as pneumonia, septicemia, urinary tract and soft tissue infections are caused by the Gram-negative bacterium Klebsiella pneumonia (Wu et ai., 2015). Another most pathogenic bacteria Pseudomonas sp. infect our body organs like liver, brain, bones, and sinuses. *Escherichia coli* is a colon Gram-negative pathogenic bacterium that causes several diseases viz. intestinal sickness, bloody diarrhea, bloody urine, pale skin, decreased urine output, bruising dehydration, biofilm formation, and pro-inflammatory interleukin-8 secretion, respectively (Ellis *et al.* 2020). *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are Enterobacteriaceae which is Carbapenem-resistant reported in worldwide (Bassetti *et al.* 2019). Neonatal sepsis and pneumonia are the dangerous disease in human beings caused by Streptococci. Among them, Streptococcus mutans causing dental caries disease have their surface protein antigens acting as a virulence factors (Nakano *et al.* 2008). The bacterium, *Staphylococcus aureus causes* blood infections, nosocomial infections and surgical wound infections (Haque *et al.* 2018).

2. Fungal diseases

Fungi are responsible to causing several serious infectious diseases of human which differ fundamentally from other microbial infections in diverse ways. Based on the host's

immunological status, multiple tissues of a patient may get infected. The invasive mycoses caused by fungi impact human health. The GAFFI (Global Action Fund for Fungal Infections) focus the devastating impact of focal fungal diseases of intact immune systems acquired human being. Fungal keratitis affect on human eyes causing blind disease more than one million in each year reported by GAFFI (GAFFI -2018; Kneale *et al.* 2016). Fungi are responsible for causing serious infectious fungal disease such as *Histoplasma capsulatum* and Mucormycetes (endemic dimorphic fungi), *Aspergillus*, *Candida*, *Cryptococcus* species, *Pneumocystis jirovecii etc.*., 2012).

3. Antiviral diseases

Virus causes many serious infections of human and other organisms due to their changeable of genomic characters and diversity. The pandemics situation arise in the world due to deadly viruses which increased the risk of spreading viral diseases between continents (Drexler,2010). New viral diseases have been reported continuously with severe health issues, and the lack of effective antiviral treatment makes them more severe (Kapoor et al. 2017). Several emerging diseases caused by viruses viz. Respiratory disease, HIV, Influenza, Herpes simplex virus (HSV), Dengue, Chikungunya, Zika, Hepatitis A (HSV), Hepatitis B (HSB), Hepatitis C (HCV), smallpox etc. (Dyer *et al.* 2015; Monath *et al.* 2016). At present, the world's population affected by Coronavirus disease (COVID-19) caused by a newly identified coronavirus has become pandemic (Ghildiyal *et al.* 2020). Human immunodeficiency virus (HIV) affect the immune system which is known as acquired immune deficiency syndrome (AIDS) (Read, 2018). Viruses found various strategies of invasion of viruses found on host cells due to their unique configuration of surface molecules, efficient replication and genetic variation linked to the host resources (Bekhit *et al.* 2014). The cell- mediated and innate immunity system involves preventing the entry of viruses in the body.

Drug resistance in bacteria: the alarming need for new antibiotics

Antibiotics are the drug used to significant controlled of infectious diseases of human and various animals. Pests and pathogens in crop field also controlled by the application of different types of antibiotics. The pathogenic microbes have become resistance uncertainly against some common antibiotics which dependent on the non-judicial use and the level of antibiotic consumption (Zaman *et al.* 2017). At present, the antimicrobial resistance (AMR) bacteria causing many serious infections which has now captured global attention (Golkar et al. 2014). Therefore, novel antibiotics are desperately needed for battling these rapidly evolving pathogens. WHO priority drug-resistant pathogens and their current antibiotics of choice for their treatment are shown in Table 1.

Table 1: WHO global priority MDR pathogens and their currently useing antibiotics (2017).

Priority category	Pathogens	Antibiotic resistance	Grams stain	Currently using antibiotics	References
Critical	*Acinetobacter baumannii*	Carbapenem-resistant	Gram-negative	Colistin, carbapenems, sulbactam, rifampin and tigecycline	Viehman *et al.* 2014
	Pseudomonas aeruginosa	Carbapenem-resistant	Gram-negative	Ticarcillin-clavulanate, ceftazidime, aztreonam, imipenem, ciprofloxacin and colistin	Kanj and Kanafani 2011
	Enterobacteriaceae, ESBL-producing Enterobacteriaceae	Carbapenem-resistant	Gram-negative	Polymyxins, fosfomycin, carbapenems, tigecycline and aminoglycosides	Morrill *et al.* 2015
	Klebsiella pneumonia	Third generation cephalosporin-resistant	Both Gram-positive and Gram-negative	Cefepime, Ceftriaxone, Ceftazidime, Rifampin Clindamycin, Ampicillin Vancomycin, Teicoplanin	Moghnieh *et al.* 2015
	Escherichia coli				
	Enterobacter spp.				
	Serratia spp.				
	Proteus spp.				
	Providencia spp.				
	Morganella spp.				
High	*Enterococcus faecium*	Vancomycin-resistant	Gram-positive	Streptogramin, linezolid, daptomycin, oritavancin and tigecycline	Linden 2002
	Staphylococcus aureus	Methicillin-resistant	Gram-positive	Vancomycin, trimethoprim-sulfamethoxazole, clindamycin, linezolid, tetracyclines and daptomycin	LaPlante *et al,* 2008

		Vancomycin intermediate and resistant		Tigecycline, Chloramphenicol, Erythromycin, Rifampin, Teicoplanin	Zhu et al.2015
	Helicobacter pylori	Clarithromycin-resistant	Gram-negative	Amoxicillin, esomeprazole, rabeprazole, omeprazole, metronidazole, levofloxacin and clarithromycin	Kali 2015
	Campylobacter	Fluoroquinolone-resistant	Gram-negative	Erythromycin, ciprofloxacin and fluoroquinolones	Safavi *et al.* 2016
	Salmonella spp.	Fluoroquinolone-resistant	Gram-negative	Ciprofloxacin,Levofloxacin, Gatifloxacin, moxifloxacin Ofloxacin	Cuypers *et al.* 2018
	Neisseria gonorrhoeae	Third generation cephalosporin-resistant	Gram-negative	Sulfonamides, Penicillin, Tetracyclines and Quinolones	Bala *et al.* 2010
		Fluoroquinolone-resistant		Ciprofloxacin	Espinosa et al.2015
Medium	*Streptococcus pneumoniae*	Penicillin-non-susceptible	Gram-positive	Penicillin	Pinto *et al.* 2019
	Haemophilus influenzae	Ampicillin-resistant	Gram-negative	Ampicillin	Maddi *et al.* 2017
	Shigella spp.	Fluoroquinolone-resistant	Gram-negative	Nalidixic acid; ciprofloxacin; norfloxacin; ofloxacin.	Pazhani *et al.* 2008

Plant metabolites as potential antimicrobials against MDR strains

Continuous searching of novel antimicrobial compounds is an important line of research due to antibiotic resistance acquired by several microorganisms. Plants are the potent source of antimicrobial compounds. Various plant secondary metabolites like alkaloids including phenylalkylamines, pyrrolidines, pyrrolizidines, tropanes and purine alkaloids, several flavonoids and tannins, alcoholic, aldehydal, phenolic, ketonic and esterified derivatives of terpenoids, quinines and resins have antimicrobial properties against many fungi and bacteria.

1. Antibacterial agents

Many plant derived compounds of different metabolite class have antibacterial efficacy. Some of the potential metabolites and their specific antibacterial activity are shown in Table 3 and also shown in Figure 1.

Table 3: Antibacterial activity of secondary metabolites produced from medicinal plants**.**

S. No.	Lead molecules	Plant sources	Target microorganisms	Mode of action	References
1.	Anolignan B	*Terminalia sericea* Burch. ex DC. (Family: Combretaceae)	*B. subtilis, E. coli, K. pneumoniae, S. aureus*	Potential to selectively inhibit cyclooxygenase COX-1	Eldeen *et al.* 2006
2.	(a) Benzoquinone (b) Benzopyran	*Gunnera perpensa* L**.** (Family: Gunneraceae)	*B. cereus, Cryptococcus neoformans, S. aureus, S. epidermis*	(a) Catalase enzyme inhibition (b) DNA Gyrase-B Inhibition	Drewes *et al.* 2005; Ghaneya *et al.* 2008
3.	(a) Carnosol (b) 7-O-methylepirosmanol	*Salvia chamelaeagnea* K. Bergius. (Family: Lamiaceae)	*B. cereus, S. aureus*	Leakage of cellular components	Kamatou *et al.* 2007
4.	Diterpene and coumaric acids	*Baccharis grisebachii* Hieron. (Family: Asteraceae)	*S. aureus*	Disruption of bacterial cell membrane and binding to bacterial genomic DNA to inhibit cellular functions, ultimately leading to cell death	Feresin *et al.* 2003; Lou *et al.* 2012
5.	Ferruginol	*Juniperus excelsa* M. Bieb. (Family: Cupressaceae)	*M. smegmatis, M. intracellulare, M. chelonae, M. xenopi*	Potentiates oxacillin activity by acting as an efflux pump inhibitor	Topçu *et al.* 1999; Smith *et al.* 2007

6.	Flavonoids	*Combretum erythrophyllum*, (Burch.) Sond. (Family: Combretaceae) *Morus alba* L., *Morus mongolica* Schneider (Family: Moraceae) *Broussnetia papyrifera* (L.) Vent (Family: Moraceae) *Sophora favescens* Ait. (Family: Leguminosae) *Echinosophora koreensis* Nakai (Family: Leguminosae)	*B. subtilis*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus*, *S. aureus*, *Salmonella typhimurium*, *Staphylococcus aureus*, *S. epidermis*	Inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, alteration of the membrane permeability, and attenuation of the pathogenicity	Martini *et al.* 2005; Sohn *et al.* 2004; Xie *et al.* 2015
7.	Gingerols	*Zingiber officinale* Rosc. (Family: Zingiberaceae)	*M. avium, M.tuberculosis*	Disruption of the cell wall of bacteria causing cytoplasmic leakage	Hiserodt *et al.* 1998; Park *et al.* 2008
8.	Glucosinolates	*Aurinia sinuata* (L.) Griseb. (Family: Brassicaceae)	*Aeromonas hydrophila*, *B. cereus*, *Chryseobacterium indologenes*, *Clostridium perfrigens*, *E. faecalis*, *Enterobacter sakazakii*, *E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *Micrococcus luteus*, *P. aeruginosa*, *Pseudomonas*	Disruption of the bacterial cell membranes	Blažević *et al.* 2010; Borges *et al.* 2015

			luteola, *S. aureus*, *Vibrio vulnificus*		
9.	Helihumulone	*Helichrysum cymosum* (L.) D. Don (Family: Asteraceae)	*B. cereus*, *B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*	---	Mathekga *et al.* 2000
10.	Methyl gallate and gallic acid	*Rhus chinensis* Mill. (Family: Anacardiaceae)	*Actinomyces viscosus*, *Lactobacillus casei*, *L. acidophilus*, *L. salivarus*, *Porphyromonas gingivalis*, *Streptococcus mutans*, *S. sobrinus*, *Salmonella* spp.	DNA gyrase or ATPase inhibition	Kang *et al.* 2008; Choi *et al.* 2014
11.	Naphtoquinones	*Tabebuia avellanedae* Lorentz ex Griseb. (Family: Bignoniaceae)	*S. aureus*	Bacterial membrane binding and intracellular ROS generation leading to apoptosis	Machado *et al.* 2003; Ravichandran *et al.* 2019
12.	Quassinoides	*Ailanthus altissima* (Mill.) Swingle (Family: Simaroubaceae)	*Mycobacterium tuberculosis*	---	Rahman *et al.* 1997
13.	Sesquiterpenoid	*Warburgia salutaris* (Bertol.f.) Chiov. (Family: Canellaceae)	*B. subtilis*, *E. coli*, *K. pneumoniae*, *S. aureus*	Inactivation of MurA enzyme of peptidoglycans biosynthesis	Rabe and van Staden, 1997; Bachelier, 2006
14.	Terpenoids	*Spirostachys Africana* Sond. (Family: Euphorbiaceae)	*E. coli*, *S. aureus*	Loss of cellular membrane integrity	Mathabe *et al.* 2006; Guimarães *et al.* 2019

15.	3,5,7-Trihydroxy-flavone (galangin)	*Helichrysum aureonitens* Sch. Bip. (Family: Asteraceae)	*Bacillus cereus*, *B. subtilis*, *Micrococus kristinae*, *Staphylococcus aureus*	Disruption of the integrity of cytoplasmic membrane producing loss of potassium	Afoyalan and Meyer, 1997; Cushnie and Lamb, 2005
16.	Xanthones	*Canscora decussata* Schult. (Family: Gentianaceae)	*M. tuberculosis*	Molecular oligomerization of these amphipathic molecules is crucial in the disruption of the bacterial inner membrane and in causing bacterial death	Ghosal and Chaudhary, 1975; Yasunaka *et al.* 2005; Koh *et al.* 2018

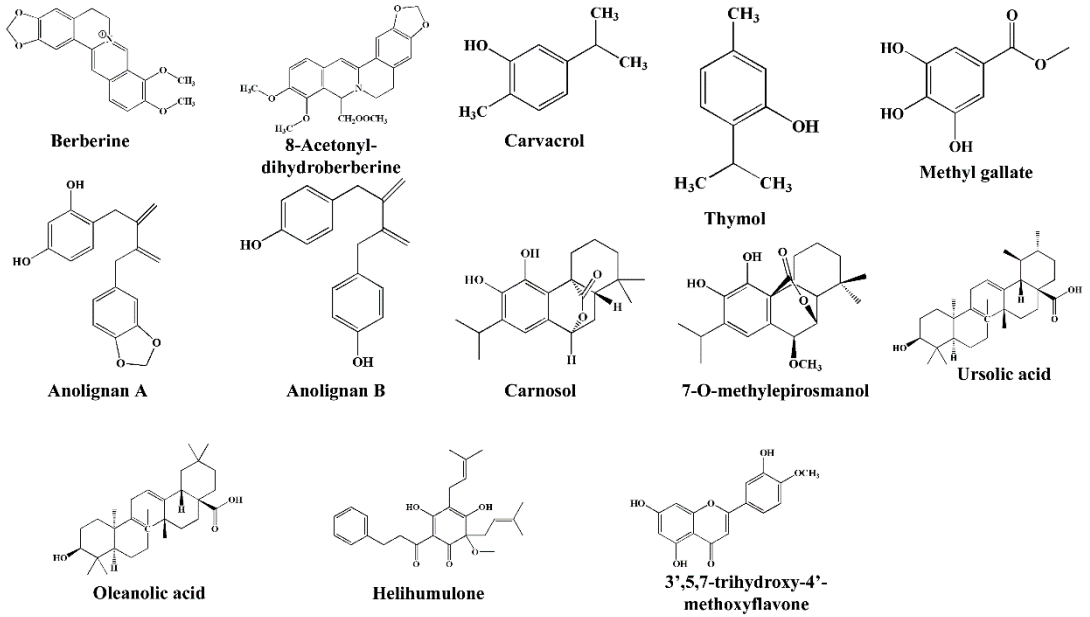


Figure 1: Some potential phytochemicals used agains the MDR bacteraial strains

2. Antifungal agents

There are several antifungal compounds of various plant metabolite classes which have been isolated and identified from different plants. There are several antifungal compounds such as dimethyl pyrrole, hydroxydihydrocornin-aglycones, indole derivatives etc. of various metabolite classes which have been identified and isolated from different plants (Schultes, 1978). The clinically available antifungal agents may be classified into three natural product classes and four synthetic chemicals classes. More than 600 plants having antifungal properties have been reported but most of which remains unexplored in terms of isolation and identification of the particular antifungal compounds (Arif *et al.* 2011). Some of the potent active antifungal agents derived from various plants are mentioned in Table 4 and also shown in Figure 2.

Table 4: Plant-derived antifungal compounds and target pathogens

Phytochemicals	Plant (part)	Target	Mode of action	References
Pinosylvin (Phenolic compound)	*Pinus* spp. (Family: Pinaceae, Gymnosperm)	*C. albicans* and *Saccharomyces cerevisiae*	Adsorption and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation	Lee *et al.* 2005
4-methoxy-5,7-dihydroxyflavone 6-Cglucoside (isocytisoside)	Leaves and stems of *Aquilegia vulgaris* L. (Family: Ranunculaceae)	*Aspergillus niger*	Decreases oxygen consumption in conidial germination and disrupting the integrity of the plasma membrane	Bylka *et al.* 2004; Cotoras *et al.* 2011
3,4',5,7-tetraacetyl quercetin (Flavonoid)	Wood of *Adina cordifolia* (Roxb.) Benth. & Hook.f. ex B.D.Jacks. (Family: Rubiaceae)	*Aspergillus* fumigatus and *Cryptococcus neoformans*	Enhances the disruption of the plasma membrane and mitochondrial dysfunction; and inhibiting cell wall formation, cell division, protein synthesis, and the efflux-mediated pumping system	Rao *et al.* 2002; Aboody and Mickymaray, 2020

Scopoletin (Hydroxycoumarin)	Seed kernels of *Melia azedarach* L. (Family: Meliaceae)	*Fusarium verticillioides, Candida tropicalis*	Interferes with the synthesis of essential fungal cell components and is able to disrupt both cell wall and plasma membrane; and reduces preformed biofilms and its proliferation	Carpinella *et al.* 2005; Lemos *et al.* 2020
CAY-1 (triterpene saponin)	*Capsicum annuum* L. (Family: Solanaceae)	*Candida* spp, *Aspergillus fumigatus*	Disruption of the membrane integrity of fungal cells	Renault *et al.* 2003
smilagenin 3-O-β-D-glucopyranoside (Spirostanol saponin)	Roots of *Smilax medica* Schltdl. & Cham. (Family: Smilacaceae)	*Candida albicans, C. glabrata* and *C. tropicalis*	---	Sauton *et al.* 2006
6-α-O-β-D-xylopyranosyl-(1-->3)-β-D-quinovopyranosyl-(25R)-5α-spirostan-3β,23α-ol	*Solanum chrysotrichum* Schltdl. (Family: Solanaceae)	*Aspergillus niger* and *Candida albicans*	Possess a detergent effect that diminishes superficial tension, causing cell-wall damage and fungal-cell disintegration	Zamilpa *et al.* 2002; Herrera-Arellano *et al.* 2013
jujubogenin 3-O-α-l-arabinofuranosyl(1-->2)-[β-D-glucopyranosyl(1-->3)]-α-larabinopyranoside	Stems of *Anomospermum grandifolium* Eichler (Family: Menispermaceae)	*C. albicans*	Weakens the virulence and kills fungi through destroying the cell membrane	Plaza *et al.* 2003; Zhang *et al.* 2006
Dioscin	Rhizomes of *Dioscorea cayenensis* Lam. (Family: Dioscoreaceae)	*C. albicans, C. glabrata* and *C. tropicalis*	Membrane-disruptive mechanism	Sauton *et al.* 2004
Tigogenin-3-O-β-D-glucopyranosyl (1-->2)-[β-D-	*Tribulus terrestris* L. (Family:	*C. neoformans* and *C. krusei*	Cell membrane disruption	Zhang *et al.* 2005

xylopyranosyl (1-->3)]-βD-glucopyranosyl (1-->4)-β-D-galactopyranoside	Zygophyllaceae)			
1,7-dihydroxy-4-methoxyxanthone	*Securidaca longipedunculata* Fresen. (Family: Polygalaceae)	*Aspergillus niger*, *A. fumigatus*, and *Penicillum* species	Effect on sterol biosynthesis by reducing the amount of ergosterol	Joseph *et al.* 2006; Pinto *et al.* 2011
3-methoxysampangine (Alkaloid)	*Cleistopholis patens* (Benth.) Engl. & Diels (Family: Annonaceae)	*C. albicans*, *A. fumigatus*, and *C. neoformans*	---	Sloodnikova *et al.* 2004

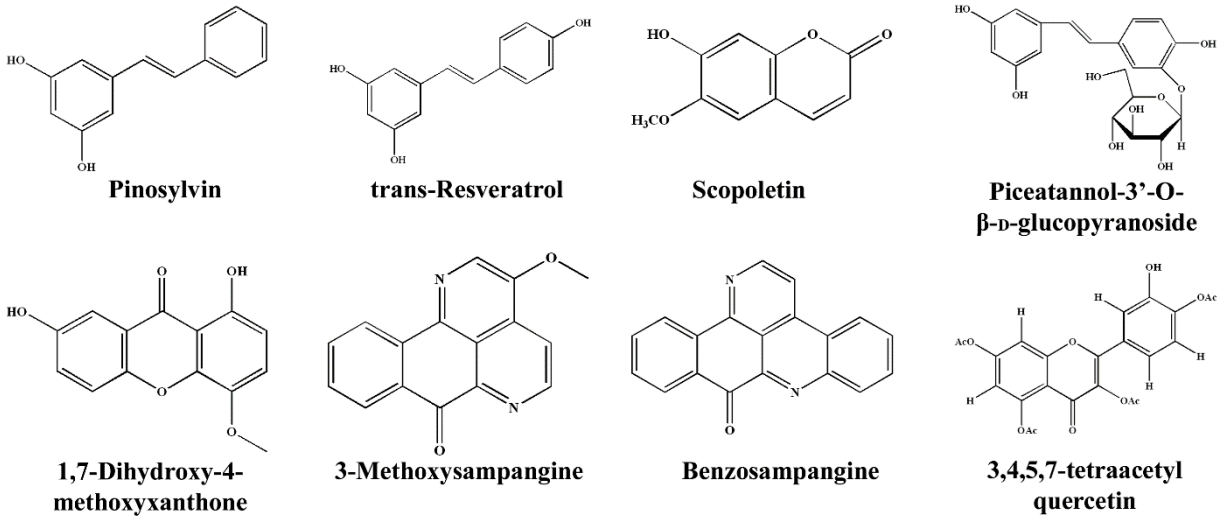


Figure 2: Some potential phytochemicals used against the MDR fungal strains

3. Antiviral agents

Medicinal plants are potent sources of antiviral agents with some advantages over conventional synthetic antiviral drugs to combat the global disease burden mainly due to viral infections by flaviviridae, herpesviridae and picornaviridae family in terms of their broad therapeutic potency and limited side effects (Sohail *et al.* 2011). An insight into the Indian medicinal plant derived antiviral compounds and their potential role in the treatment of viral infections is demonstrated in Table 5 and also shown in Figure 3.

Table 5: Indian medicinal plants having antiviral potencies

Plant (part)	Phytochemicals	Target	Mode of action	References
Artocarpus lakoocha Roxb. (Family: Moraceae)	Oxyresveratrol	HSV-1, HSV-2	Inhibitory activity at the early and late phase of viral replication of HSV-1 and HSV-2. Inhibition of late protein synthesis	Chuanasa *et al.* 2008
Citrus reticulata Blanco (Family: Rutaceae)	Tangeretin and nobiletin (Polymethoxylated flavones)	RSV	Affected the intracellular replication of RSV. Tangeretin down regulated the expression of RSV phosphoprotein (P protein)	Scaglia *et al.* 2014
Curcuma longa L. (Family: Zingiberaceae)	Curcumin	HIV	Inhibition of HIV-1 integrase	Barthelemy *et al.* 1998
Humulus lupulus L.(Family: Cannabaceae)	Xanthohumol (Chalcone)	BVDV	---	Zhang *et al.* 2010
Papavar somnifera Linn. (Family: Papavaraceae)	Papaverine	HIV	Interfere with the envelope precursor protein gp 120 of HIV	Turano *et al.* 1989
Phyllanthus emblica Linn. (Family: Euphorbiaceae)	---	HIV	Inhibition of HIV reverse trasncriptase	Mekkawy *et al.* 1995
Saraca indica Linn. (Family: Fabaceae)	---	HIV	HIV protease inhibition	Kusumoto *et al.* 1995
Swietenia macrophylla King	Limonoids (Lignin)	HCV	---	Wu *et al.* 2012; Cheng *et al.* 2014

(Family: Meliaceae)				
Terminalia arjuna (Roxb. ex DC.) Wight & Arn. (Family: Combretaceae)	---	HIV	HIV protease inhibition	Kusumoto *et al.* 1995
Terminalia chebula Retz. (Family: Combretaceae)	---	HIV	Inhibition of HIV reverse transcriptase, inhibition of viral adsorption to cells	Nonaka *et al.* 1990; Weaver *et al.* 1992; Mekkawy *et al.* 1995
Ziziphus jujuba Mill. (Family: Rhamnaceae)	Jubanines (Alkaloids)	PEDV	----	Kang *et al.* 2015

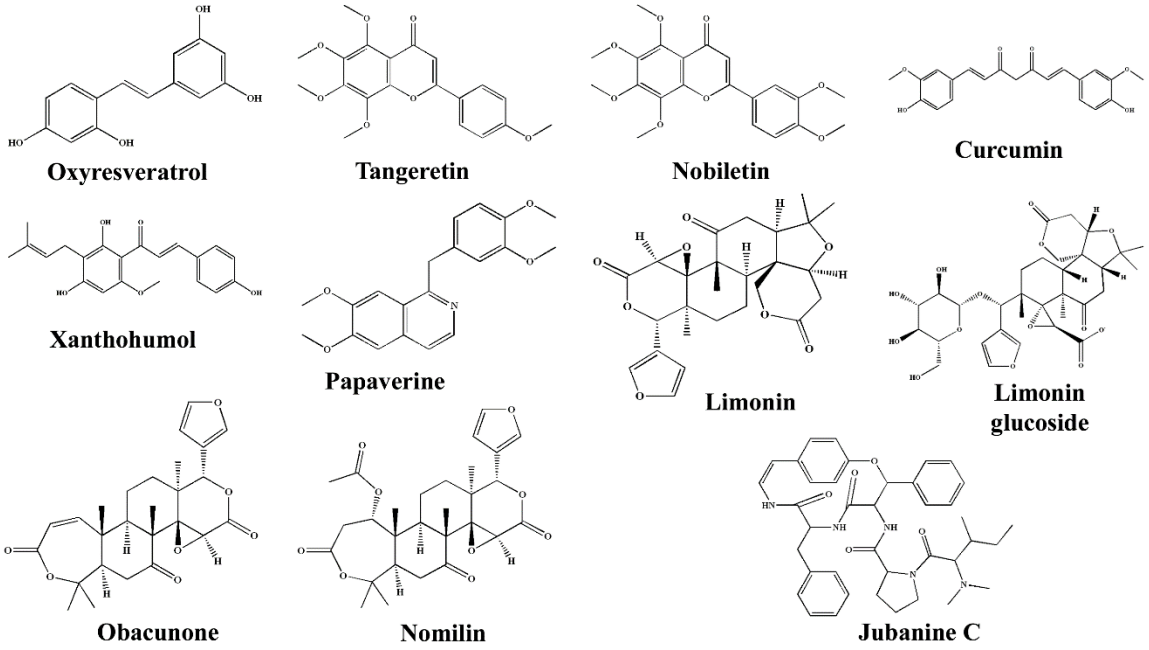


Figure 3: Some potential phytochemicals used against the viral strains.

Mechanism of antimicrobial activity of plant antimicrobials

Various mechanisms of antimicrobial activity of plant antimicrobials generally include damaging the bacterial cell membrane, inhibiting Efflux Pumps (EPs), inhibiting DNA and protein biosynthesis and also synergistic effects in combination with conventional antibiotic drugs leading to a direct impact on restoring microbial efficacy in resistant strains (Khameneh *et al.* 2019). The detailed mode of action of the plant antimicrobials are described under the following headings. Mechanism of antibacterial activity, Inhibition of biofilm formation and Efflux pump (EP) inhibitors, Attenuating bacterial virulence, Synergism between phytochemicals

Table 6: Effective concentrations of phytochemicals inhibiting efflux pumps of bacterial strains.

Plants	Active Compound	Effective concentration	Drug resistant bacteriatested	Targeted efflux pumps	References
Acer saccharum Marshall (Family: Aceraceae)	Catechol	5 and 10 mg/ml	*P. aerugenosa* ATCC 15692 and UCBPPPA14, *E. coli* ATCC 700928, *P. mirabilis* HI4320	EtBr EP	Maisuria *et al.* 2015
Alkanna orientalis (L.) Boiss. (Family: Boraginaceae)	Sarothrin	100µM	*Staphylococcus aureus* NCTC 8325-4	NorA	Bame *et al.* 2013
Alpinia galanga (L.) Willd. (Family: Zingiberaceae)	1′-S-1′-acetoxyeugenol acetate	3.12–25 mg/L	*Mycobacterium smegmatis* mc2 155	EtBr EP	Roy *et al.* 2012
Ammannia baccifera L.(Family: Lythraceae)	4-hydroxy-α-tetralone	125 µg/ml	MDR *E. coli*	YojI	Dwivedi *et al.* 2014
Berberis vulgaris L. (Family: Berberidaceae)	Berberine and Palmatine	250–1,000 µg/ml and 50–100 µg/ml	MDR *P. aeruginosa* (clinical isolates)	MexAB-OprM	Aghayan *et al.* 2017
Capsicum annuum L. (Family: Solanaceae)	Capsaicin	25 µg/ml	*S. aureus* SA-1199B	NorA	Kalia *et al.* 2012
Catharanthus roseus (L.) G. Don (Family: Apocynaceae)	Catharanthine	25 mg/L	*P. aeruginosa*	EtBr EP	Dwivedi *et al.* 2017

Chenopodium Ambrosioides L. (Family: Chenopodiaceae)	Essential oil	170.6 µl/ml	*Staphylococcus aureus* IS-58	Tet(K) EP	Limaverde *et al.* 2017
Cuminum cyminum L. (Family: Apiaceae)	Cumin-methanol extract	5 mg/ml	*S. aureus* MRSA OM505	LmrS	Kakarla *et al.* 2016
Eucalyptus tereticornis Sm. (Family: Myrtaceae)	Ursolic acid and derivatives	25 and 50 µg/ml	MDR *E. coli* (KG4)	AcrA/B-TolC, MacB and YojI	Dwivedi *et al.* 2014a
Holarrhena antidysenterica (L.) Wall. ex A. DC. (Family: Apocynaceae)	Conessine	20 mg/L	*P. aeruginosa*	MexAB-OprM	Siriyong *et al.* 2017
Hypericum olympicum L. (Family: Hypericaceae)	Olympicin A	50µM	*S. aureus* 1199B	NorA	Shiu *et al.* 2013
Ipomoea muricata (L.) Jacq. (Family: Convolvulaceae)	Lysergol	10 µg/ml	*E. coli* MTCC1652 and KG4	YojI	Maurya *et al.* 2013
Persea lingue (HY) Nees ex Kopp (Family: Lauraceae)	Kaempferol rhamnoside	1.56 mg/L	*S. aureus* SA-1199B	NorA	Holler *et al.* 2012
Salvia fruticosa Mill. (Family: Lamiaceae)	Essential oil	5 µl/ml	*S. epidermidis* (clinical isolates)	Tet(K) EP	Chovanova *et al.* 2015
Scutellaria baicalensis Georgi (Family: Lamiaceae)	Baicalein	16 µg/ml	*S. aureus* SA-1199B	NorA	Chan *et al.* 2011
Terminalia chebula Retz. (Family: Combretaceae)	Gallotannin	12.1–97.5 µg/ml	MDR uropathogenic *E. coli*	EtBr EP	Bag and Chattopadhyay, 2014
Wrightia tinctoria R. Br. (Family: Apocynaceae)	Indirubin	1.25 and 2.5 mg/L	*S. aureus* SA-1199B	NorA	Ponnusamy *et al.* 2010

Mechanism of antifungal activity of plant antimicrobials

The antifungal compounds exert its impacts on the pathogenic fungal cells by any of the six different mechanisms *i.e.*,Inhibition of cell wall formation, Cell membrane disruption, Dysfunction of the fungal mitochondria, Inhibition of cell division, Inhibition of RNA/DNA synthesis or protein synthesis, and Inhibition of efflux pumps which are described in the following paragraphs(Freiesleben and Jäger 2014).The mechanism of action is being elaborated below:

1. Inhibition of cell wall formation

Phenolic compounds exert their antifungal action through inhibition of cell wall formation (Davidson and Taylor, 2007). The cell wall integrity is disrupted due to the inhibition of the synthesis of the cell wall components β-glucans and chitin (Myers, 2006; Walker and White, 2011). Lectins (naturally occurring proteins found in most plants serve a protective function for plants) interfere with the biosynthesis of chitin and inhibit cell wall formation (Smith *et al.* 2008; Pusztai, 2012). Naphtoquinone also acts through interference with the fungal cell wall however, the exact mechanism is yet not known (Mc Clanahan, 2009). In general, the sulfur compounds show hindrance tothe polysachharide as well as cell wall formation in fungal cells (Sousa *et al.* 2012).

2. Cell membrane disruption

Ergosterols are the essential components of the fungal cell membrane. The antifungal drugs target either to bind with these sterols or to inhibit its synthesis (Walker and White, 2011). Thus, the cell membrane's integrity is disrupted and the membrane becomes leaky. The fungicidal effect of phenylpropanoid eugenol of clove essential oil results due to impaired biosynthsis of ergosterol that causes direct damage to the cell membrane leaving a lesion (Pinto *et al.* 2009). The monoterpenes and the sesquiterpenes act as antifungal compounds through cell membrane disruption. The saponins (saikosaponins) and steryl glycosides have detergent like property which acts on cell membrane disruption. They anchor with their lipophilic moiety in the lipophilic membrane bilayer after complexing with cholesterol, and the hydrophilic moiety outside the cell (Morrissey and Osbourn, 1999; Ashour and Wink, 2011). Essential oils are typical lipophilic and disrupt the membrane structure while passing through it (Bakkali *et al.* 2008). Lipophilic terpenoids of tea tree essential oil exert damage of membrane and make it permeable (Jung *et al.* 2007). Phenolic compounds, terpenoid alcohols and polyacetylenes also have a direct action on the biomembrane (Pinto *et al.* 2006; Tian *et al.* 2012). Studies assumed that the main mechanism of the flavonoid papyriflavonol A and polygodial is the cell membrane disruption but it did not confirm how it disrupted the membrane (Wu *et al.* 2009). Nitrogenous compounds may cause cell membrane disruption by inhibition of ergosterol biosynthesis, or its complexing (Bagiu *et al.* 2012).

3. Dys function of the fungal mitochondria

Inhibition of the mitochondrial electron transport will result in reduction in mitochondrial membrane potential leading to apoptosis or subsequent programmed cell death of the target cells (Mc Clanahan, 2009). The phenolic compounds, phenylpropanoids, monoterpenes and the sesquiterpenes can destroy fungal mitochondria. Essential oil from dill seeds (*Anethum graveolens* L., family Apiaceae), containing mainly carvone, limonene monoterpenes and a

phenylpropanoid apiol impacts on mitochondrial dysfunction by reactive oxygen species (ROS) accumulation, inhibition of electron transport or proton pump and inhibition of ATPase activity in the mitochondria (Tian *et al.* 2012). While the sesquiterpene polygodial isolated from *Polygonum punctatum* Elliott var. *punctatum* of family Polygonaceae targets the mitochondrial ATP synthase, polyphenol plagiochin E shows antifungal activity through ROS accumulation (Lunde and Kubo, 2000; Wu *et al.* 2008). The flavonoid baicaleinshows antifungal activity byaffecting the mitochondrial homeostasis, without elevating the intracellular ROS level (Kang *et al.* 2010). The sulfur containing compounds of leaves of Ramson plant (*Allium ursinum* L., family Amaryllidaceae) act as uncouplers of proton translocation in the mitochondrial membrane and hamper ADP phosphorylation (Sousa *et al.* 2012).

4. Inhibition of cell division

Most of the antifungal compounds derived from plants inhibit cell division by inhibiting the formation of the mitotic spindle through arresting the microtubule polymerization process (Walker and White, 2011). A polyphenol reservatrol produced in various nut plants arrests the cell cycle at the S-phase and thereby affects fungal cell growth inhibiting cell division processes in the tested fungus (Davidson and Taylor, 2007).

5. Inhibition of RNA/DNA synthesis or protein synthesis

The antifungal phytochemical agents enter the fungal cells via active transport by ATPases, and interfere with the DNA transcription resulting in faulty RNA synthesis (Mc Clanahan, 2009). The naphtoquinones act through two different mechanisms; *i.e.*, interference with mRNA transcription and interference with the protein synthesis (Van Der Weerden *et al.* 2010). The sulfur compounds with some specific functional groups interfere with the fungal membrane integrity, inhibit synthesis of DNA, RNA, proteins and stop the production or activity of associated enzymes (Sousa *et al.* 2012).

6. Inhibition of efflux pumps

Efflux pumps transport toxic substances and accumulated drugs out of the fungal cells. Drug resistance developed by over expression of efflux pumps can be reduced by inhibiting them using plant based phytochemicals. Anthraquinone inhibits the energy-dependent efflux pumps (Kang *et al.* 2010) while Carvacrol, a terpenoid alcohol, activates specific signaling pathways that results in calcium stress and inhibits the target of rapamycin pathway of nutrient sensing and exerted dose dependent inhibition on yeast growth (Rao *et al.* 2010).

Mechanism of antiviral activity of phytochemicals

Plant derived antiviral compounds mainly functions as inhibitors of replication and terminators of RNA and protein synthesis. Following are some mechanisms of action of some phytochemicals derived from medicinal plants used as antiviral compounds (Kapoor *et al.* 2017).

Alkaloids: Alkaloids block virus binding and inhibit the virus growth. It reduces the viral titers in lungs in case of HIV, HSV.

Flavonoids: Flavonoids have inhibitory effect on reverse transcriptase and blocks RNA synthesis in HIV, HSV and Influenza.

Lectins: Lectins act by arresting reverse transcriptase and N-glycohydrolase activity and thus inhibit HIV virus penetration in human.

Polyphenols: Polyphenols inhibit the viral cell entry by modulating the viral surface structure and effects the expression of virus proteins on cell surface.

Polysaccharides: These compounds inhibit viral replication and viral binding to host cell.

Proteins: GAP31 (Gelonium anti-HIV protein of 31 kDa) anti-HIV plant proteins that are isolated from the medicinal plants *Momordica charantia* (Family: Cucurbitaceae) and *Gelonium multiflorum* A.Juss. (Family:), inhibit viral DNA integration and viral replication. Panaxagin inhibits reverse transcriptase and inhibit viral protein synthesis in HIV.

Terpenes: Saponins inhibit the replication in HSV, Influenza viruses.

Conclusion and future prospects of drug discovery in pharmaceuticals

The antibacterial activity of phytochemicals is a well-known phenomenon and in recent years, knowledge regarding these bioactive compounds has widely increased. Many mechanisms of antimicrobial action have been suggested. The purpose of this study is to investigate the prospect of antimicrobials of plant origin and to study the correlation between the type of natural compounds and its antifungal mechanism of action. Generally phytochemicals cause damage to the bacterial membrane, suppression of some virulence factors, including enzymes and toxins, and inhibition of bacterial biofilm formation. Many antiviral crude and purified compounds successfully obtained from various plants which have efficacy toward different viruses. Fungal infection of human cure by photoactive secondary metabolites derived from medicinal plants. Hence, antimicrobial phytochemicals have widely been used as preventative and curative therapeutic solutions against multi-drug-resistant pathogens. Some plant-based antimicrobials have antibiotic resistance modifying activities besides their direct antimicrobial activity. These compounds have the synergistic effect in combination with conventional antibiotics at lower MIC dose against drug resistant bacteria. From this review, it has been evidenced that some secondary metabolites were proven to be potent antimicrobial agents against drug resistant pathogens which mainly include bacteria, fungi and virus. The findings show that it is difficult to simplify the mechanism of antimicrobial action of plant secondary metabolites as there is an array of bioactivities of a particular phytochemical compound. There are some correlations between few secondary metabolite classes and its antimicrobial mechanism of action, but many of the compounds act through more than one mechanism of action. Hence, it is necessary to study the mechanism of action of each bio-molecule instead of a group of compounds.

The pharmaceutical companies are engaged in developing more powerful antimicrobials from medicinal plant-derived natural products which have diverse chemical structures and novel mechanisms of action to control antibiotic resistance by MDR pathogens. With the emergence of antibiotics resistance developed by microbial strains create a serious problem to combat against different infectious diseases. In this situation, there is an urgent need to engage scientists of different research back grounds to handle with thousands of phytochemicals for their antimicrobial activity in vitro as well as in vivo against all types of pathogenic organisms. Medicinal plant derived secondary metabolites including alkaloids, flavonoids, tannins, and terpenoids have shown antimicrobial potentialities against several MDR microbial pathogens (Figure 4). Botanists, microbiologists, ethno-pharmacologists and natural productchemists should work in combination on utilization of these natural products and structural modification either synthetically or via biotransformation. The finding of antimicrobial compounds with

sufficiently lower minimum inhibitory concentrations (MICs), little toxicity, and optimum bioavailability for efficient and safe use in humans and animals is the biggest challenges. The isolation of such antimicrobials from thousands of bio-molecules in plant bodies is tough and laborious. The application of sophisticated tools viz. column purification, high performance liquid chromatography coupled to mass spectrometry (HPLC–MS), liquid chromatography–mass spectrometry (LC–MS), liquid chromatography–nuclear magnetic resonance–mass spectrometry (LC–NMR–MS), capillary NMR (cap-NMR) spectroscopy, LC–solid phase extraction (SPE)–NMR along with bioassay-guided fractionation and high-throughput bioassays will accelerate the access of plant-derived natural products (Lahlou, 2013). The green synthesis of various metallic nano-particles from medicinal plants which act against MDR pathogens is a recent trend in searching novel antimicrobial agents. Hence, more budgetary allocation by the government and multinational pharmaceutical companies in research and development of such plant based antimicrobials is necessary.

References:

1. Afoyalan AJ, Meyer JJM (1997) The antimicrobial activity of 3, 5, 7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. J Ethno 57: 177-81.
2. Aminov RI (2010) A brief history of the antibiotic era: lessons learned and challenges for the future. Front Micro. ;1:134. PMCID: PMC3109405.
3. Ang JY, Ezike E, Asmar BI. (2004) Antibacterial resistance. Ind J Pediatr;71:229-39.
4. Arif T, Mandal TK, Dabur R. (2011) Natural products: anti-fungal agents derived from plants. Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry: 283-311. ISBN: 978-81-308-0448-4.
5. Armstead J, Morris J, Denning DW (2014) Multi-Country Estimate of Different Manifestations of Aspergillosis in Cystic Fibrosis. PLoS ONE 9(6): e98502. https://doi.org/10.1371/journal.pone.0098502
6. Asokan GV., Ramadhan U, Ahmed E, Sanad H, (2019) WHO Global Priority Pathogens List: A Bibliometric Analysis of Medline-PubMed for Knowledge Mobilization to Infection Prevention and Control Practices in Bahrain, Oman Med J. ; 34(3): 184–193. doi: 10.5001/omj.2019.37
7. Bachelier A, Mayer R, Klein CD (2006) Sesquiterpene lactones are potent and irreversible inhibitors of the antibacterial target enzyme MurA. Bio Med Ch Let 16: 5605-09.
8. Bala M, Sood S (2010) Cephalosporin Resistance in *Neisseria_gonorrhea*, J Glob Infect Dis. 2(3): 284–290. doi: 10.4103/0974-777X.68537
9. Ballal M, Devadas S, Chakraborty R, Shetty V (2014). Emerging trends in the etiologyand antimicrobial susceptibility pattern of enteric pathogens in rural coastalIndia. Int J Clin Med, 5:425-32.
10. Bandow JE, Brötz H, Leichert LIO, Labischinski H, and Hecker M (2003) Proteomic Approach to Understanding Antibiotic Action. Antimicrobial Agents and Chemotherapy, 47: 948-955. DOI: 10.1128/AAC.47.3.948-955.2003
11. Barthelemy S, Vergnes L, Moynier M., Guyot D, Labidalle S, Bahraoui E (1998) Curcumin and curcumin derivatives inhibit Tat-mediated transactivation of type 1 human immunodeficiency virus long terminal repeat. ResVirol 149(1): 43-52.

12. Bassetti M, Peghin M, Vena A and Giacobbe DR (2019) Treatment of Infections Due to MDR Gram-Negative Bacteria. Front. Med. 6:74. doi: 10.3389/fmed.2019.00074.
13. Beceiro A, Tomas M, Bou G (2013) Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev; 26:185.
14. Bekhit A E D, & Bekhit A A (2014) Natural Antiviral Compounds. Studies in Natural Products Chemistry, 195–228. doi:10.1016/b978-0-444-63281-4.00007-0
15. Bender J K, Cattoir V, Hegstad K, Sadowy E, Coque T M, Westh H, Werner G (2018). Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: towards a common nomenclature. Drug Resistance Updates. doi:10.1016/j.drup.2018.10.002
16. Blažević I, Radonić A , Mastelić J, Zekić M, Skocibušić M, Maravić A (2010) Glucosinolates, glycosidically bound volatiles and antimicrobial activity of *Aurinia sinuata* (Brassicaceae). Food Ch 121: 1020-28.
17. Borges A, Abreu AC, Ferreira C, Saavedra MJ, Simões LC, Simões M (2015) Antibacterial activity and mode of action of selected glucosinolate hydrolysis products against bacterial pathogens. *J Food Sci Technol*. 2015;52(8):4737-4748. doi:10.1007/s13197-014-1533-1.
18. Borges AJ, Saavedra M , Simoes M (2015) Insights on antimicrobial resistance, biofilms and the use of phytochemicals as new antimicrobial agents, Curr. Med. Chem., 22 (21) pp. 2590-2614.
19. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. *Sci Transl Med* ;4(165):165rv13. doi:10.1126/scitranslmed.3004404
20. Bylka W, Szaufer M, Matlawska J, Goslinska O (2004) Antimicrobial activity of isocytisoside and extracts of *Aquilegia vulgaris.* L Appl. Microbiol 39(1): 93-7. doi: 10.1111/j.1472-765X.2004.01553.x.
21. Carpinella MC, Ferrayoli CG, Palacios SM (2005) Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from *Melia azedarach* L. fruits. J Agric Food Chem 53(8): 2922-27.
22. Chandra P, Sharma V, Kant S (2018) From Commodity to Brand: The Country of Origin Branding Perspective for Indian Medicinal and Aromatic plants. Bus Strat Dev.1–9. https://doi.org/10.1002/bsd2.31
23. Cheng YB, Chien YT, Lee JC, Tseng CK, Wang HC, *et al.* (2014) Limonoids from the seeds of *Swietenia macrophylla* with inhibitory activity against Dengue virus 2. J Nat Prod 26: 2367-2374.
24. Choi J-G, Mun S-H, Chahar HS, Bharaj P, Kang O-H, *et al.* (2014) Methyl gallate from Galla rhois successfully controls clinical isolates of *Salmonella* infection in both *In Vitro* and *In Vivo* systems. PLoS ONE 9(7): e102697. doi:10.1371/journal.pone.0102697.
25. Chuanasa T, Phromjai J, Lipipunc V, Likhitwitayawuid K, Suzuki M, *et al.* (2008) Anti-*herpes simplex* virus (HSV-1) activity of oxyresveratrol derived from Thai medicinal plant: Mechanism of action and therapeutic efficacy on cutaneous HSV-1 infection in mice. Antiviral Res 80: 62-70.

26. Cotoras M, Mendoza L, Muñoz A, Yáñez K, Castro P, Aguirre M (2011) Fungitoxicity against *Botrytis cinerea* of a flavonoid isolated from *Pseudognaphalium robustum*. Molecules 16: 3885-95.
27. Cowan MM (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12:564–582
28. Cushnie TPT, Lamb AJ (2005) Antimicrobial activity of flavonoids. Int J Antimicrob Agents: 26: 343–56.
29. Cuypers W L, J Jan, W Vanessa, . Klemm EJ, D Stijn , Puyvelde S V, (2018) Fluoroquinolone resistance in *Salmonella*: insights by whole-genome sequencing, Microb Genom, 4(7), https://doi.org/10.1099/ mgen.0.000195.
30. Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433
31. Dhama K,Tiwari R,Chakraborty S,Saminathan M, Kumar ,Karthik K, Rahal (2014) Evidence based antibacterial potentials of medicinal plants and herbscountering bacterial pathogens especially in the era of emerging drug resistance: an integrated update, Int. J. Pharmacol. 10 ,1–43.
32. Drewes SE, Khan F, van Vuuren SF, Viljoen AM (2005) Simple 1, 4-benzoquinones with antibacterial activity from stems and leaves of *Gunnera perpensa*. Phytochem 66:1812-16.
33. Drexler J F, Gloza-Rausch F, Glende J, Corman V M, Muth D, Goettsche M, Drosten C (2010) Genomic Characterization of Severe Acute Respiratory Syndrome-Related Coronavirus inEuropean Bats and Classification of Coronaviruses Based on Partial RNA-Dependent RNA Polymerase Gene Sequences. Journal of Virology, 84(21), 11336–11349. doi:10.1128/jvi.00650-10
34. Du D, Wang-Kan X, Neuberger A, van Veen H W, Pos K M, Piddock L J *et al.* (2018). Multidrug efflux pumps: structure, function and regulation. Nat. Rev. Microbiol. 16, 523–539. 10.1038/s41579-018-0048-6
35. Dwivedi G R, Tyagi R, Sanchita Tripathi S, Pati S, Srivastava S K, *et al.* (2017). Antibiotics potentiating potential of catharanthine against superbug *Pseudomonas aeruginosa*. J. Biomol. Struct. Dyn. 1–15. doi: 10.1080/07391102.2017.1413424.
36. Dyer O (2015) Zika virus spreads across Americas as concerns mount over birth defects. BMJ. 351:h6983. doi:10.1136/bmj.h6983
37. EdeogaHO, OkwuDE, MbaebieBO, (2005) Phytochemical constituents of someNigerian medicinal plants, Afr. J. Biotechnol. 4 (7) 685–688.
38. Efferth T, Koch E (2011) Complex interactions between chemicals. The multi-target therapeutic concept of phytotherapy. Curr Drug Targets 12:122–132
39. El Chakhtoura NG, Saade E, Iovleva A, Yasmin M, Wilson B, Perez F, & Bonomo R A (2018). Therapies for multidrug resistant and extensively drug-resistant non-fermenting gram-negativebacteria causing nosocomial infections: a perilous journey toward "molecularly targeted" therapy. Expert Review of Anti-Infective Therapy, 16(2), 89–110.
40. Eldeen IMS, Elgorashi EE, Mulholland DA, van Staden J (2006) Anolignan B a bioactive compound from the roots of *Terminalia sericea*. J Ethno 103: 135-38.

41. Ellis SJ, Crossman LC, McGrath, CJ *et al.* (2020) Identification and characterisation of enter aggregative *Escherichia coli* subtypes associated with human disease. *Sci Rep* 10, 7475 https://doi.org/10.1038/s41598-020-64424-3
42. Epand R M, Walker C, Epand R F, & Magarvey N A (2016). Molecular mechanisms of membrane targeting antibiotics. Biochimica et Biophysica Acta (BBA) Biomembranes, 1858(5), 980–987. doi:10.1016/j.bbamem.2015.10.018
43. Epand RF, Sarig H , Mor A , Epand RM, (2009) Cell-wall interactions and the selective bacteriostatic activity of a miniature oligo-acyl-lysyl, Biophys. J., 97 pp. 2250-2257
44. Epand RF, Sarig H , Mor A , Epand RM, (2009) Cell-wall interactions and the selective bacteriostatic activity of a miniature oligo-acyl-lysyl, Biophys. J., 97 pp. 2250-2257, https://doi.org/10.1016/j.bpj.2009.08.006
45. Espinosa K, Park JA, Gerrity JJ, Buono S, Shearer A, Dick C Mak M L, Teramoto K, Klausner J D, Pandori M, Hess D (2015) Fluoroquinolone Resistance in *Neisseria gonorrhoeae* After Cessation of Ciprofloxacin Usage in San Francisco, Sexually Transmitted Diseases. 42 (2) 57-63 doi: 10.1097/OLQ.0000000000000233
46. Fabio A, Cermilli C, Fabio PA, Nicoletti P and Quaglio P (2007). Screening of the antibacterial effect of a variety of essential oils on micro organism responsible for respiratory infection. Phytotherapy Research 21 374-377. doi: 10.1002/ptr.1968.
47. Fadli M, Saad A, Sayadi S, Chevalier J, Mezrioui NE, Pagès JM, *et al.* (2012) Antibacterial activity of *Thymus maroccanus* and *Thymus broussonetii* essential oils against nosocomial infection-bacteria and their synergistic potential with antibiotics. Phytomedicine 19: 464-71.
48. Feresin GE, Tapia A, Gimenez A, Ravelo AG, Zacchino S, Sortino M, Schmeda-Hirschmann, G (2003) Constituents of the Argentinian medicinal plant *Baccharis grisebachii* and their antimicrobial activity. J Ethno 89: 73- 80.
49. Founou RC, Founou LL, Essack SY (2017) Clinical and economic impact of antibiotic resistance in developing countries: A systematic review and meta-analysis PLoS One 12(12): e0189621. doi: 10.1371/journal.pone.0189621.
50. Friedman N D, Temkin E, Carmeli Y (2016) The negative impact of antibiotic resistance. Clinical Microbiology and Infection, 22(5), 416–422. doi:10.1016/j.cmi.2015.12.002
51. GAFFI. Global Fund for Fungal Infections [Internet]. (2018). Available from: https://www.gaffi.org.
52. Ghildiyal R, Prakash V, Chaudhary VK, Gupta V, Gabrani R (2020) Phytochemicals as Antiviral Agents: Recent Updates. In: Swamy M. (eds) Plant-derived Bioactives, DOIhttps://doi.org/10.1007/978-981-15-1761-7_12
53. Ghosal S, Chaudhary RK (1975) Chemical constituents of Gentianaceae XVI: antitubercular activity of *Canscora decussata* Schult. J Phar Sc 64: 888–89.
54. Global Action Fund for Fungal Infections (GAFFI). (2017) Priority Fungal Infections. Available online: http: //www.gaffi.org/media/fact-sheets/ .
55. Golkar Z, Bagazra O, Pace DG (2014) Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. J Infect Dev Ctries 8(2):129-36.doi: 10.3855/jidc.3573.

56. Guimarães A, Meireles L, Lemos M, Guimarães M, Endringer D, Fronza M Scherer R (2019) Antibacterial activity of terpenes and terpenoids present in essential oils. Molecules. 24: 2471. 10.3390/molecules24132471.
57. Hall C W, Mah T-F, (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria, *FEMS Microbiology Reviews*, 41,(3) 276–301, https://doi.org/10.1093/femsre/fux010
58. Haque M, Sartelli M, McKimm J, Abu Bakar M (2018). Health care-associated infections - an overview. Infect Drug Resist. 15;11:2321-2333. doi: 10.2147/IDR.S177247.
59. Hassan GS, Farag NA, Hegazy GH, Arafa RK (2008) Design and synthesis of novel benzopyran-2-one derivatives of expected antimicrobial activity through DNA gyrase-b inhibition. Arch Pharm Chem Life Sci 341: 725 –33. Doi: 10.1002/ardp.200700266.
60. Herrera-Arellano A, López-Villegas EO, Rodríguez-Tovar AV, *et al.* (2013) Use of antifungal saponin SC-2 of *Solanum chrysotrichum* for the treatment of vulvovaginal candidiasis: *in vitro* studies and clinical experiences. A J T Comp Alt Med 10(3): 410-417.
61. Hiserodt RD, Franzblau SG, Rosen RT (1998) Isolation of 6-, 8-, and 10-gingerol from ginger rhizome by HPLC and preliminary evaluation of inhibition of *Mycobacterium avium* and *Mycobacterium tuberculosis*. J Ag Food Ch 46: 2504–08.
62. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance ofbacterial biofilms. Int J Antimicrob Agents;35:322-32.
63. Jassim S A A, & Naji MA (2003) Novel antiviral agents: a medicinal plant perspective. Journal of Applied Microbiology, 95(3), 412–427. doi:10.1046/j.1365-2672.2003.02026.x
64. Joseph CC, Moshi MJ, Sempombe J, Nkunya MHH (2006) (4-methoxy-benzo [1, 3] dioxol-5-yl)-phenylmethanone: an antibacterial benzophenone from *Securidaca longepedunculata*. Afr J Trad CAM 3(3): 80-86.
65. Jun-Jie K, Shuimu L, Yang B, Wendy WLS, Thet TA, Jianguo L, Verma C, Konstantin P, Roger WB, Shouping L (2018) Antimicrobial activity profiles of amphiphilic xanthone derivatives are a function of their molecular oligomerization. BBA - Biomem 1860: 2281–98.
66. Kali A (2015) Antibiotics and bioactive natural products in treatment of methicillin resistant Staphylococcus aureus: a brief review. Pharmacogn Rev 9:29–34
67. Kamatou GPP, van Vuuren SF, van Heerden FR, Seaman T, Viljoen AM (2007) Antibacterial and antimycobacterial activities of South African *Salvia* species and isolated compounds from *S. chamelaeagnea*. S A J Botany 73: 552- 57.
68. Kang KB, Ming G, Kim GJ, Ha TKQ, Cho Hi, *et al.* (2015) Cyclopeptide alkaloids from the roots of *Ziziphus jujuba.* Phytochem 43: 264-267.
69. Kang M, Oh J, Kang I, Hong S, Choi C (2008) Inhibitory effect of methyl gallate and gallic acid on oral bacteria. J Microbiol 46: 744-50.
70. Kanj SS, Kanafani ZA (2011) Current concepts in antimicrobial therapy against resistant Gram-negative organisms: extendedspectrum b-lactamase-producing enterobacteriaceae, carbapenem-resistant enterobacteriaceae, and multidrug-resistant *Pseudomonas aeruginosa*. Mayo Clin Proc 86:250–259

71. Kapoor R, Sharma B, Kanwar SS (2017) Antiviral Phytochemicals: An Overview . Biochem Physiol 6:220. doi:10.4172/2168-9652.1000220
72. Karageorgopoulos DE, Wang R, Yu XH , Falagas ME (2012) Fosfomycin: evaluation of the published evidence on the emergence of antimicrobial resistance in Gram-negative pathogens, J. Antimicrob. Chemother, 67(2):255-268. doi:10.1093/jac/dkr466
73. Kneale M, Bartholomew SJ, Emma D and Denning W D (2016) Global access to antifungal therapy and its variable cost; J Antimicrob Chemother ; 71: 3599 –3606 doi:10.1093/jac/dkw 325.
74. Kong K, Schneper L, Mathee K (2010) Beta-lactam antibiotics: from antibiosis toresistance and bacteriology. APMIS;118(1):1-36. doi:10.1111/j.1600-0463.2009.02563.x
75. Kostakioti M, Hadjifrangiskou M, Hultgren SJ. (2013) Bacterial biofilms: develop-ment, dispersal, and therapeutic strategies in the dawn of the postantibioticera. Cold Spring Harb Perspect Med, 3(4):a010306.
76. Kourtesi C, Ball AR, Huang Y, Jachak SM, Vera DM, Khondkar P, *et al.* (2013) Mi-crobial efflux systems and inhibitors: approaches to drug discovery and thechallenge of clinical implementation. Open Microbiol J;7:34-52.
77. Kumar S, Varela MF, (2013) Molecular mechanisms of bacterial resistance to antimicrobial agents. Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.) http://www.formatex.info/microbiology4/vol1/522-534.
78. Kusumoto IT, Nakabayashi T, Kida H, Miyashiro H, Hattori M, Namba T, Shimotohno K (1995) Screening of various plant extracts used in ayurvedic medicine for inhibitory effects on human immunodeficiency virus type 1 (HIV-1) protease. Phytother Res 9: 180-184.
79. LaPlante KL, Leonard SN, Andes DR, Craig WA, Rybak MJ (2008) Activities of clindamycin, daptomycin, doxycycline, linezolid, trimethoprim-sulfamethoxazole, and vancomycin against community-associated methicillin-resistant *Staphylococcus aureus* with inducible clindamycin resistance in murine thigh infection and in vitro pharmacodynamic models. *Antimicrob Agents Chemother*.;52(6):2156-2162. doi:10.1128/AAC.01046-07.
80. Lee SK, Lee HJ, Min HY, Park EJ, Lee KM, Ahn YH, Cho YJ, Pyee JH (2005) Antibacterial and antifungal activity of pinosylvin, a constituent of pine. Fitoterapia 76(2): 258-60. doi: 10.1016/j.fitote.2004.12.004.
81. Lemos ASO, Florêncio JR, Pinto NCC, Campos LM, Silva TP, Grazul RM, Pinto PF, Tavares GD, Scio E, Apolônio ACM, Melo RCN and Fabri RL (2020) Antifungal activity of the natural coumarin scopoletin against planktonic cells and biofilms from a multidrug-resistant *Candida tropicalis* strain. Front Micro 11: 1525. doi: 10.3389/fmicb.2020.01525.
82. Linden PK (2002) Treatment options for vancomycin-resistant enterococcal infections. Drugs 62:425–441
83. Lou Z, Wang H, Rao S, Sun J, Chaoyang M, Li J (2012) P-Coumaric acid kills bacteria through dual damage mechanisms. Food Control 25: 550–54. 10.1016/j.foodcont.2011.11.022.
84. Lowy FD (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. JCl in Investig; 1; 111(9): 1265–1273.doi: 10.1172/JCI18535.

85. Machado TB, Pinto AV, Pinto MCFR, Leal ICR, Silva MG, Amaral ACF, Kuster RM, Netto-dos-Santos KR (2003) *In vitro* activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicillin-resistant *Staphylococcus aureus*. In J Antimicrobial Age 21: 279-84.
86. Maddi S, Kolsum U, Jackson S, Barraclough R, Maschera B, Simpson KD, Pascal TG, Durviaux S, Hessel EM, Singh D (2017) Ampicillin resistance in *Haemophilus influenzae* from COPD patients in the UK. Int J Chron Obstruct Pulmon Dis.12:1507-1518. doi: 10.2147/COPD.S135338.
87. Mahboubi M, Bidgoli FG (2010) Antistaphylococcal activity of *Zataria multiflora* essential oil and its synergy with vancomycin. Phytomed 17: 548-50.
88. Martini ND, Katerere DRP, Eloff JN (2004) Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). J Ethno 93: 207-12.
89. Mathabe MC, Nikolova RV, Lall N, Nyazema NZ (2006) Antibacterial activities of medicinal plants used for the treatment of diarrhoea in Limpopo Province, South Africa. J Ethno 105: 286-293.
90. Mathekga ADM, Meyer JJM, Horn MM, Drewes SE (2000) An acylated phloroglucinol with antimicrobial properties from *Helichrysum caespititium*. Phytochem 53: 93-96.
91. Mekkawy SE, Meselhy MR, Kusumoto IT, Kadota S, Hattori M, Namba T (1995) Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriptase. Ch Phar Bull 43(4): 641-648.
92. Mishra A, Sharma A K, Kumar S, Saxena A K, & Pandey AK (2013) *Bauhinia variegate* Leaf Extracts Exhibit Considerable Antibacterial, Antioxidant, and Anticancer Activities. BioMed Research International, 2013, 1–10. doi:10.1155/2013/915436
93. Moghnieh R, Estaitieh N, Mugharbil A, Jisr T, Abdallah D I, Ziade F, Sinno L, Ibrahim A (2015) Third generation cephalosporin resistant *Enterobacteriaceae* and multidrug resistant gram-negative bacteria causing bacteremia in febrile neutropenia adult cancer patients in Lebanon, broad spectrum antibiotics use as a major risk factor, and correlation with poor prognosis, Front. Cell. Infect. Microbiol.,5(11),| https://doi.org/10.3389/fcimb.2015.00011
94. Mohammed SAA, Suresh M (2020) Anti-fungal efficacy and mechanisms of flavonoids. Antibiotics 9: 45. doi:10.3390/antibiotics9020045.
95. Monath TP, Woodall JP, Gubler DJ, Yuill TM, Mackenzie JS, , Martins RM, Reiter P, Heymann D L (2016) Yellow fever vaccine supply: A possible solution. Lancet 387: 1599-1600. doi:10.1016/S0140-6736(16)30195-7
96. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL (2015) Treatment options for carbapenem-resistant enterobacteriaceae infections. Open Forum Infect Dis 2:ofv050
97. Nakano K, Nomura R, & Ooshima T (2008). Streptococcus mutans andcardiovascular diseases. Japanese Dental Science Review, 44(1), 29–37. doi:10.1016/j.jdsr.2007.09.001.
98. Nelson (1982) The comparative clinical pharmacology and pharmacokinetics of vindesine, vincristine, and vinblastine in human patients with cancer, Pediatr. Blood Cancer 10(2)115–127.

99. Nitha B, Remashree AB, and Balachandran I (2012) Antibacterial Activity of Some Selected Indian Medicinal Plants. Int J Pharm Sci Res, Vol. 3(7): 2038-2042.
100. Nonaka GI, Nishioka I, Nishizawa M, Yamagishi T, Kashiwada Y, Dutschman GE, Bodner AJ, Kilkuskie RE, Cheng YC, Lee KH (1990) Anti-AIDS agents, 2: inhibitory effects of tannins on HIV reverse transcriptase and HIV replication in H 9 lymphocyte cells. J Nat Prod 53: 587-595.
101. Pandey AK, Kumar S (2013) "Perspective on plant products as antimicrobial agents: a review," Pharmacologia, 4, pp. 469–480.
102. Park, Miri & Bae, Jungdon & Lee, Dae-Sil. (2008) Antibacterial activity of gingerol isolated from ginger rhizome against periodontal bacteria. Ph Res PTR 22: 1446-49. 10.1002/ptr.2473.
103. Pazhani G P, Niyogi S K, Singh A K, Sen B, Yamasaki S, Taneja N, Kundu M. Shinji Y, Ramamurthy T (2008). Molecular characterization of multidrug-resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India. Journal of Medical Microbiology, 57(7), 856–863. doi:10.1099/jmm.0.2008/000521-0
104. Pendleton NJ, Gorman SP, Gilmore BF (2013). Clinical relevance of the ESKAPEpathogens. Expert Rev Anti Infect Ther;11(3):297-308.
105. Peterson E, Kaur P (2018) Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens, Front Microbiol ; 9: 2928. doi: 10.3389/fmicb.2018.02928
106. Piddock LJ (2006) Multidrug-resistance efflux pumps? Not just for resistance, Nat. Rev. Microbiol. (4): 629–636.
107. Pinto E, Afonso C, Duarte S, Vale-Silva L, Costa E, Sousa E, Pinto M (2011) Antifungal Activity of xanthones: evaluation of their effect on ergosterol biosynthesis by high-performance liquid chromatography. Ch Bio Drug Des 77: 212-22. 10.1111/j.1747-0285.2010.01072.x.
108. Pinto TCA, Neves FPG, Souza ARV, Oliveira LMA, Costa NS, Castro LFS, Mendonça-Souza CRdV, Peralta JM and Teixeira LM (2019) Evolution of Penicillin Non-susceptibility Among *Streptococcus pneumoniae* Isolates Recovered From Asymptomatic Carriage and Invasive Disease Over 25 years in Brazil, 1990–2014. Front. Microbiol. 10:486. doi: 10.3389/fmicb.2019.00486

SPONDIAS MOMBIN: ETHNOBOTANICAL PROPERTIES AND CANCER MANAGEMENT

Olufunke Christy Akanji[1], Sayed Mohammed Firdous[2*], Sourav Pal[3] and MacDonald Idu[4]

[1]Adekunle Ajasin University, Akunga-Akoko, Ondo State, Nigeria

[2]Department of Pharmacology, Calcutta Institute of Pharmaceutical Technology & AHS, Uluberia, Howrah: 711316, West Bengal, India

[3]P. G. Institute of Medical Sciences, Dhurabila, Dhamkuria, Chandrakona Town, Paschim Medinipur: 721201, West Bengal, India

[4]Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State, Nigeria

*Corresponding author E-mail: Email: firdous.oncology@gmail.com

Abstract:

Herbal plants and extracts have been used therapeutically for millennia, but their efficacy is currently being studied. Many of these plants contain antioxidants that protect against free radical damage and inflammation. These traits may help fight cancer. Malignancy is caused by many circumstances. Genetic mutations and alterations in cell signalling pathways that regulate cell growth and survival cause this molecularly. Extrinsic and intrinsic apoptotic processes are critical for cell survival and tissue homeostasis. Dysregulation of these pathways promotes cell survival, apoptosis resistance, and tumour growth and metastasis. Researchers are studying apoptotic pathways to develop cancer therapies. Cancer researchers are searching for these molecular targets and developing drugs to address them. Hog plum or yellow mombin is a tropical fruit tree called *Spondias mombin*. It treats fever, diarrhoea, and skin conditions in traditional medicine. Recent studies have shown that *S. mombin* extracts kill cancer cells via apoptosis. *S. mombin* flavonoids and polyphenols may be medicinal. Antioxidant and anti-inflammatory substances may combat cancer. Finally, *S. mombin* may treat cancer by targeting cancer cells. Identifying its active components and cancer cell molecular targets requires more research.

Keywords: Herbal plant, cancer, apoptosis, *spondias mombin*

Ethnobotany in medicine

Over the previous decades, herbal prescription has turned into a theme of worldwide significance and has been having an effect on both world well-being and global exchange. Medicinal plants keep on assuming fundamental positions in the healthcare structure of a vast percentage of the earth's total populace. This is especially valid in unindustrialized nations, where plant treatment has a lengthy and continuous record of utilization [1]. In Nigeria, the larger part of inhabitants still utilizes therapeutic plants to remedy sicknesses and call on traditional healers for their treatments [2]. Indigeneous residents generally depend on medicinal plants since these plants are great sources of chemical compositions required to battle against illnesses and afflictions within the human body. On the basis of the indigenous knowledge that has been passed down to them, people use medicinal plants to treat a variety of illnesses [3]. On

the basis of the recommendations of more experienced individuals, such as wise men, herbalists, and traditional practitioners, medicinal plants may be utilised. People have been using herbs as medicine ever since the dawn of civilization since it was believed that plants possessed healing qualities [4]. Many of the medications that are used today have been derived from natural plant sources. Currently, herbal products have been the center of attention as the essential supply of novel, nontoxic, and more efficient bioactive components with therapeutic values.

Cancer

Cancer is a malignant disease caused by uncontrolled cell division (Figure 1) to form tissues [5]. These cells can enter neighbouring organs or reach unreachable places of the body and multiply furiously, causing catastrophic sickness and death. Because they don't spread, benign tumours aren't cancerous. Cancer kinds are named after their initial cell or body place. Breast, liver, brain, bone, colon, and prostate cancers are all types of cancer. The World Health Organisation ranks cancer as the second leading cause of death (8.8 million deaths in 2015). Cancer was Africa's 7th leading cause of death in 2004 [6].

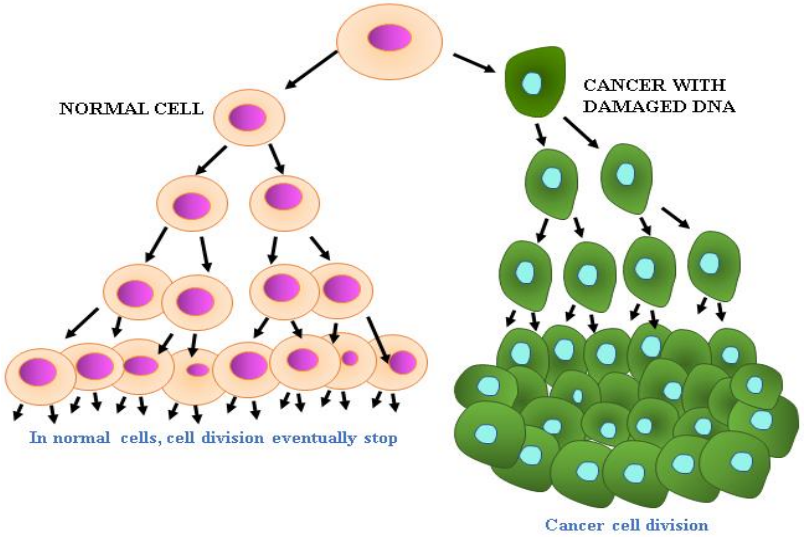


Figure 1: Uncontrolled division of cancer cell

Stages of Cancer: The phases of cancer are typically numbered from 0 to IV, with higher numbers indicating more advanced disease [7]. The specific staging criteria may vary depending on the type of cancer, but here is a general overview:

- **Phase 0:** The malignancy is still confined to the initial layer of cells, and has not metastasized to distant organs or lymph nodes.
- **Phase I:** It is localised and has not metastasized to other parts of the body.
- **Phase II:** It's more advanced and may have metastasized to lymph nodes in the area, but it hasn't moved further in the body.
- **Phase III:** Cancer has expanded to the surrounding lymph nodes and maybe adjacent tissues, but has not yet metastasized to other parts of the body.
- **Phase IV:** Metastatic cancer occurs when the disease has metastasized, or spread, to other parts of the body [8].

Causes of Cancer: There are numerous variables that can lead to cancer infected state in a sound person and are basically sub-divided into two primary categories, which are:

1) **Environmental Factors:** It's a key factor in carcinoma's pathophysiology, or how it forms. Cancer is a multifaceted illness caused by a combination of genetic and environmental factors. Among the environmental elements implicated in cancer's development are:

- **Chemicals**: The introduction of organisms to specific materials has been connected to particular sorts of cancer. These materials are named carcinogens. Tobacco smoke contains a variety of carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) and nitrosamines, which can damage DNA by forming adducts or by inducing oxidative stress. These DNA lesions can lead to mutations and genomic instability, which can promote cancer development [9].
- **Insulin resistance:** It also induces hyperinsulinemia (high levels of insulin in the blood). High amounts of insulin can encourage cell growth and division, which can aid in the development of cancer [10].

2) **Inherited Genetics**: Breast and ovarian cancers are linked to BRCA1 and BRCA2 gene mutations. However, these mutations are infrequent and account for a small percentage of breast and ovarian malignancies. Genetic alterations don't cause most breast and ovarian cancers. Lynch syndrome and familial adenomatous polyposis (FAP) also raise the risk of some cancers. Hormones and cancer-causing autoimmune disorders are affected by genetics. A study found that women with a family history of breast cancer had higher oestrogen and progesterone levels than women without a history, explaining their higher risk of breast cancer even without a breast-cancer gene [11]. African males had more prostate cancer and greater testosterone levels than European men. Overweight people had higher cancer hormone levels and greater cancer rates. Growth hormones may accelerate osteosarcoma in women who receive hormone replacement therapy [12].

The cell cycle

Malignat cells differ from normal, healthy cells in several significant ways, which contribute to their ability to grow and divide uncontrollably and to resist treatment [13]. One of the key features of cancer cells is the loss of differentiation, which means they have a less specialized structure and function compared to normal cells. This loss of differentiation can make cancer cells harder to target with drugs that rely on specific cellular functions. Cancer cells also have a self-sufficient growth signal, which means they do not require external signals to grow and divide. This can result from mutations or changes in genes involved in cell signaling pathways. Finally, cancer cells can become resistant to drugs, which can make treatment more difficult. This resistance can occur due to changes in drug metabolism, increased drug efflux, or mutations in genes involved in cell survival pathways [14]. Cancer is primarily caused by somatic cell mutations. Thus, the cell cycle (Figure 2) constitutes a change for two main processes: "Doubling" (S = synthesis) in which the DNA is synthesized and the "halving" (M = mitosis) in which cells and their content are equally divided into two cellular daughters. The periods between these processes are called gap periods (G phase) (Sandor, 2002). The stages of the cell cycle are depicted in Figure 2.

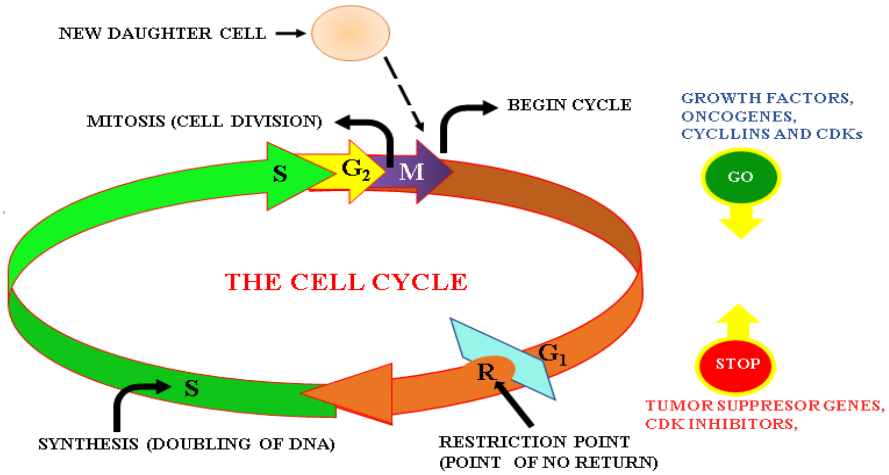


Figure 2: Cell Cycle Phase showing Control Points

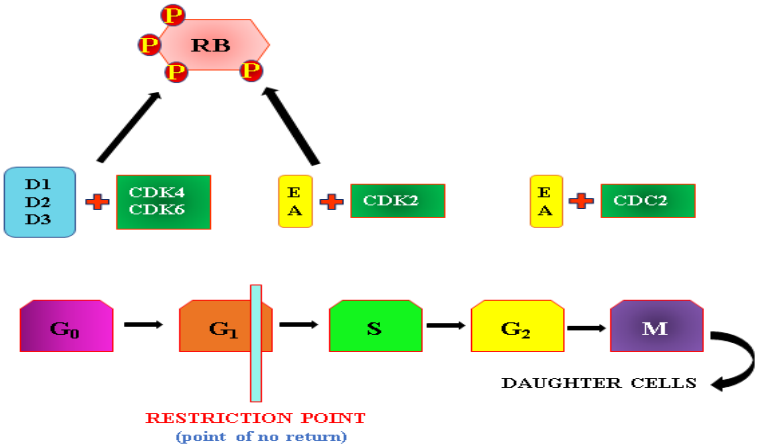


Figure 3: Progression of cell cycle.

Apoptosis

Apoptosis has been one of the most investigated procedures in biological studies since it was defined by Kerr and Harmon, in the 1991's [15]. In contrast to necrosis, this term is used to describe the state in which a cell voluntarily commits suicide in response to specific stimuli. Apoptosis is a highly controlled process that is required for optimal growth and tissue homeostasis. In cancer, abnormal cell growth and tumour formation result from a breakdown in the homeostasis between cell reproduction and cell death. As a result of research into the mechanisms of apoptosis and their dysregulation in cancer, new cancer medicines have been developed that specifically target these processes in order to cause tumour cell death [16]. Apoptosis is an extremely selective method, both physiologically and pathologically.

Mechanisms of Apoptosis: Caspases play an important role in the process of apoptosis since they can act as both initiators and executers. There are three different approaches to activating caspases. Internal (also known as mitochondrial) and extrinsic (also known as death-receptor) mechanisms of apoptosis are the two types of initiation that are typically discussed in the scientific literature [17]. Both routes eventually terminate at either a prevalent route or an apoptosis execution stage. There is a third initiation pathway that is not as well-known called the intrinsic endoplasmic reticulum pathway.

The Extrinsic Death or Receptor Pathway: When death ligands connect to their respective death receptors, the extrinsic or death receptor pathway is activated. Several death receptors have been discussed, but the type 1 TNF receptor (TNFR1), Fas (CD95/APO-1), and their ligands, TNF and Fas ligand (FasL) are the most well-known [18]. Adapter proteins like TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) and cysteine proteases like caspase 8 are used in the intracellular death domain of these death receptors. The Death-Inducing Signalling Complex (DISC) is comprised of the death ligand, the death receptor, and the adaptor protein that is recruited to the receptor upon death ligand binding [19]. DISC then triggers pro-caspase 8 assembly and activation. When activated, caspase 8 functions as a caspase initiator, cleaving other caspases to trigger apoptosis (Figure 4).

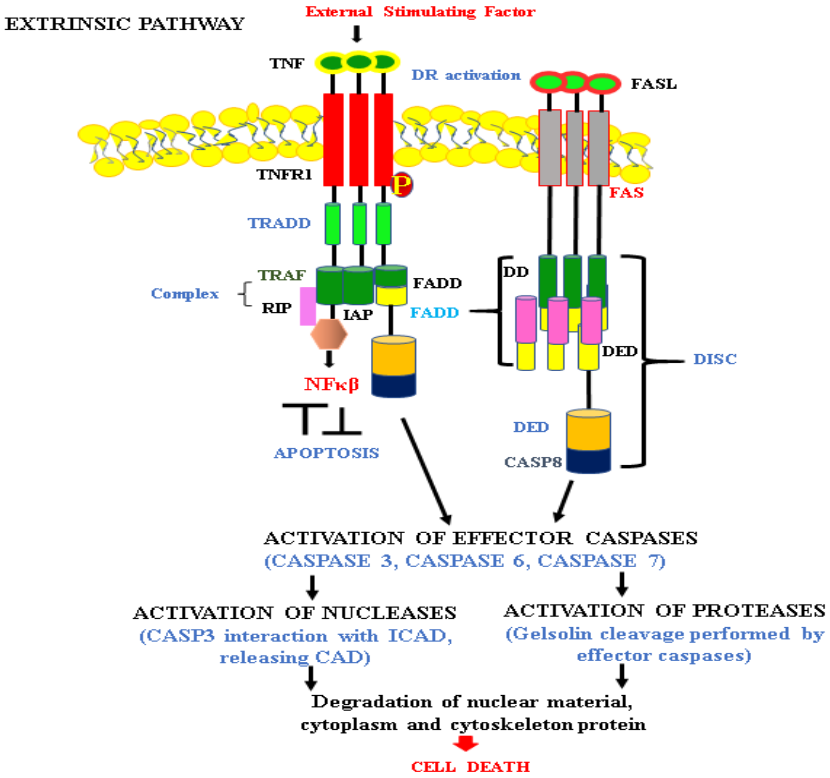


Figure 4: Downstream cascade of extrinsic pathway of cell death

The Intrinsic Mitochondrial Pathway

As its name suggests, the intrinsic route begins inside the cell. Inherited genetic damage, hypoxia, abnormally high cytosolic Ca^{2+} levels, and severe oxidative stress are all examples of internal stimuli that might initiate the intrinsic mitochondrial pathway [20]. This process results from increased mitochondrial permeability and the release of pro-apoptotic molecules like cytochrome-c into the cytoplasm and occurs regardless of the stimuli. The Bcl-2 family of proteins is responsible for the stringent regulation of this pathway; the family was called after the Bcl-2 gene, which was first identified at the chromosomal breakpoint of chromosomes 18 to 14 in non-Hodgkin follicular lymphoma [21]. There are two main classes of Bcl-2 proteins: the pro-apoptotic proteins (such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk) and the anti-apoptotic proteins (such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1). Apoptosis is regulated by antiapoptotic proteins, which prevent mitochondrial Cytochrome-C release, and by proapoptotic proteins, which increase this release (Figure 5).

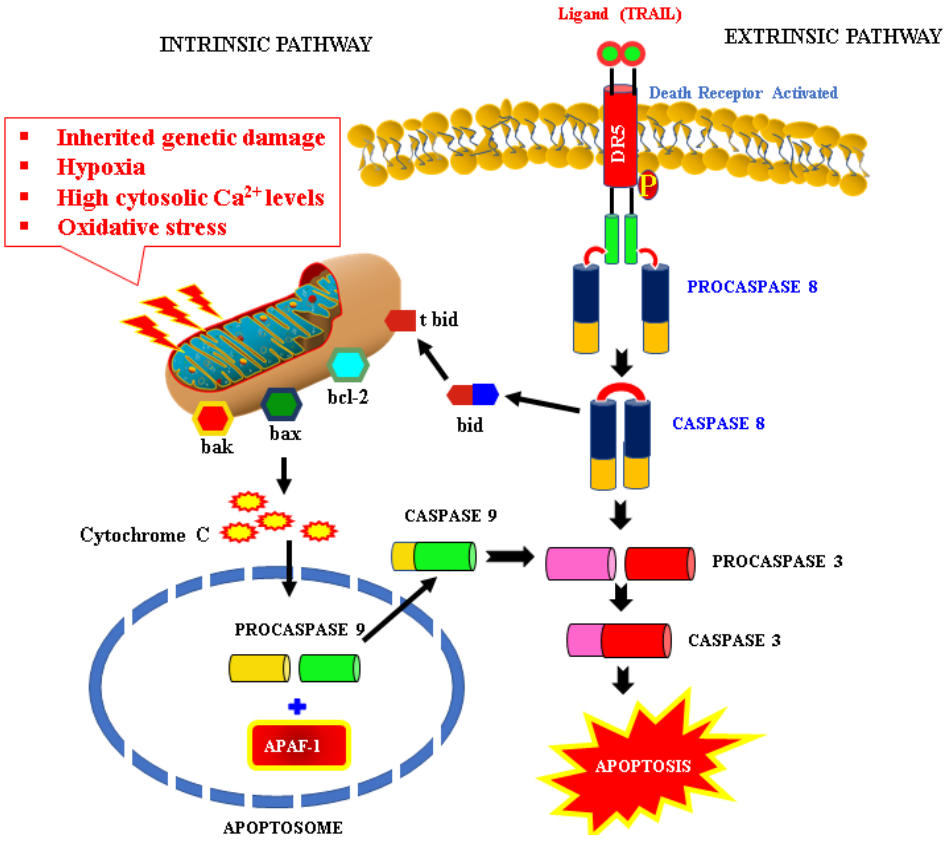


Figure 5: Downstream cascade of intrinsic pathway of cell death

Apoptosis and Carcinogenesis

A succession of genetic alterations can be traced back to cancer, and evasion of cell death is a significant modification that contributes to cancer's malignant phenotype. Kerr and Harmano (1991) established a connection between apoptosis and the removal of malignant cells, the avoidance of hyperplasia, and the inhibition of tumour growth [22]. As a result, apoptosis inhibition or resistance is crucial to cancer progression. Cancer cells can inhibit or become resistant to apoptosis in a variety of ways. There are several different ways cells can avoid apoptosis, however, they can generally be grouped into three categories:

(1) a lack of anti-apoptotic proteins or an excess of pro-apoptotic proteins,

(2) impaired caspase action, and

(3) defective death receptor signaling [23].

An imbalance between pro- and anti-apoptotic proteins

Numerous proteins with either pro- or anti-apoptotic action in cells have been identified. The ratio between pro- and anti-apoptotic proteins is more important than the absolute amount in controlling cell death. In addition, it has been found that over- or under-expression of specific genes (therefore the regulatory proteins) contributes to carcinogenesis by reducing apoptosis in cancer cells [24].

Deficiency in Capsase Activity: Caspases are still crucial to apoptosis. Low caspase concentrations or caspase activity may reduce apoptosis and cancer, which are linked to high caspase levels. Low caspase-9 levels in stage II colorectal cancer patients worsened prognoses. Devarajan *et al.* (2002) found that caspases-3 mRNA was absent or considerably reduced in breast, cervical, and ovarian cancers [25].

Targeting Apoptosis in Cancer Treatment: Targeting apoptosis in cancer treatment addresses one of cancer biology's biggest issues: cancer cells' capacity to avoid cell death. Apoptosis, which destroys damaged or aberrant cells, is strictly regulated in healthy tissues. However, cancer cells often have abnormalities in the apoptotic pathway, allowing them to avoid cell death and divide uncontrollably. Cancer treatments can remove aberrant cells and inhibit tumour growth by causing apoptosis. Apoptosis-targeted medicines may treat many malignancies, including those resistant to conventional treatments [26].

Inhibitor of Apoptosis Proteins (IAPs): IAPs regulate apoptosis, cell proliferation, and inflammation. IAPs suppress apoptosis in various malignancies and help cancer cells survive [27].

The downstream cascade of IAPs-induced caspase inhibition: Cancer cell survival and proliferation depend on molecular pathway disruption. Caspase inhibition increases NF-kB and its downstream targets, which promote cell survival and inflammation. By increasing nuclear translocation or stabilising downstream effectors like IKK and IB, IAPs can directly activate NF-kB. IAPs block caspases, upregulating NF-kB signalling and promoting cancer cell survival and proliferation. IAPs-induced caspase inhibition disrupts the MAPK signalling pathway. Caspases cleave and activate MAPK pathway components like ERK, JNK, and p38, regulating cell proliferation and differentiation. IAPs block caspases, which can cause MAPK pathway dysregulation and cancer cell proliferation and survival. IAP inhibition activates caspases and induces cancer cell death. IAP inhibitors also sensitise cancer cells to chemotherapy and radiation, boosting their efficiency [28].

XIAP (X-Linked Inhibitor of Apoptotic Proteins): X-Linked Inhibitor of Apoptotic Proteins (XIAP) is a member of the Inhibitor of Apoptosis Proteins (IAPs) family that is overexpressed in many cancers and contributes to the survival and proliferation of cancer cells by inhibiting apoptosis [29].

The downstream cascade of XIAP-induced caspase inhibition: XIAP directly binds to caspases and suppresses their activation, as well as the function of other apoptosis-related proteins like SMAC. SMAC is produced from mitochondria during apoptosis and binds to XIAP, preventing it from inhibiting caspases. Cancer cells can upregulate XIAP expression or mutations to withstand chemotherapy, radiation, and immunotherapy-induced apoptosis [30]. Thus, targeting XIAP in cancer treatment may restore cancer cell apoptosis. Small molecule inhibitors, antisense oligonucleotides, and RNA interference (RNAi) target XIAP in cancer treatment. These methods efficiently reduce XIAP expression or activity, activating caspases and inducing cancer cell death. Yamaguchi *et al.* (2005) targeted Survivin. Smac mimics target tumours with high cIAPs, while ARTS mimics target tumours with high XIAP. These findings suggest that blocking XIAP may accelerate cancer cell death, but additional research is needed to understand its mechanisms [31].

The Pro-Inflammatory Genes: Pro-inflammatory genes play a significant role in cancer treatment as they are involved in the regulation of the immune response and can impact the effectiveness of various cancer therapies, including chemotherapy, radiotherapy, and immunotherapy.

The downstream cascade of pro-inflammatory gene-induced recruitment of immune cells:

TNF, IL-1, IL-6, and IL-8 trigger immune response signalling pathways include NF-B, JAK/STAT, and MAPK. These pathways recruit and activate immune cells like T cells, natural killer cells, and macrophages in the tumour microenvironment [32]. IL-1β, IL-6, TNF-α, and CXCL8 are produced by pro-inflammatory genes and influence immune cell function and the tumour microenvironment. These cytokines can activate and recruit immune cells to the tumour location. In the tumour microenvironment, persistent inflammation recruits immunosuppressive cells including regulatory T cells and myeloid-derived suppressor cells [33]. Cancer treatment may benefit from targeting pro-inflammatory genes. Inhibiting pro-inflammatory genes can diminish tumour microenvironment inflammation and angiogenesis, limiting tumour development and invasion. Inhibiting pro-inflammatory genes reduces immunosuppression in the tumour microenvironment, which may improve immunotherapy [34].

The Tumor Necrosis Factor- Alpha (TNF-α): TNF, also known as TNF-, is a cytokine released by immune cells that may suppress cancer cell growth and cause tumour regression [35]. TNF-alpha is part of the acute phase reaction, or inflammatory response. CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons can also produce it. Activated macrophages are the main producers. TNF's main function is immune cell control. TNF, an endogenous pyrogen, can produce fever, apoptotic cell death, inflammation, and the prevention of carcinogenesis, virus replication, and sepsis through cells generating IL-1 and IL-6 [36]. Dysregulation of tumour necrosis factor has been linked to many diseases. However, TNF's cell death is minimal compared to its inflammatory mechanism. Secondary hypercalcemia and uncontrolled tumour growth have been connected to malignancy's ectopic TNF and parathyroid hormone production. After being cleaved by TNF-converting enzyme (TACE), membrane-bound pro-TNF is released. TNF has many physiological and pathological functions, including growth and immunity, inflammation, cancer development, transplant rejection, rheumatoid arthritis, and septic shock. TNF's receptors stimulate cellular signalling pathways that regulate cell survival, growth, and death. TNF's role in cancer is multifaceted. Since it may self-destruct malignant cells, it may be used to treat cancer. TNF enhances cancer cell proliferation, survival, migration, and angiogenesis in cells resistant to its cytotoxicity. Thus, TNF can promote and inhibit tumour growth [37].

The Interleukin 1 Beta (IL-1β): Pro-inflammatory cytokine IL-1β helps the immune system fight cancer. IL-1β activates various signalling pathways that govern immune cell function and tumour progression [38]. IL-1β activates NF-B downstream. IL-1β activates NF-B signalling by binding to its receptor on immune and cancer cells. This route activates cell survival, proliferation, and inflammatory genes. In cancer, NF-B pathway activation produces pro-inflammatory cytokines and chemokines that recruit immune cells to the tumour microenvironment. IL-1β activates the immune cell-regulating JAK/STAT signalling pathway. IL-1β activates the JAK/STAT pathway to boost immune cell activity, killing cancer cells. IL-1β also activates the cell survival and proliferation MAPK signalling pathway. This route promotes cell proliferation, survival, and immune cell recruitment to the tumour microenvironment by producing pro-inflammatory cytokines and chemokines [39].

The Kidney Injury Molecule 1 (KIM-1): Kidney injury molecule-1 (KIM-1) is a transmembrane protein that is increased in many cancers and may contribute to cancer aetiology. In cancer, KIM-1 activates multiple signalling pathways that regulate cell proliferation, survival, and invasion [40]. KIM-1 activates MAPK/ERK downstream. KIM-1 activates MAPK/ERK, which controls cell survival, proliferation, and migration. KIM-1 activation of this pathway promotes cell proliferation and invasion, important cancer pathogenesis processes [41]. KIM-1 activates the cell survival and proliferation pathway PI3K/Akt. KIM-1 activation of this pathway promotes cell survival and proliferation and inhibits apoptosis, which can advance cancer. KIM-1 also activates the Wnt/β-catenin signalling pathway, which controls cell proliferation and differentiation. KIM-1 activates this pathway to promote cell proliferation and invasion and suppress apoptosis [42].

Botanical Descriptions of *Spondias mombin* Linn.

Spondias mombin is a fruit-bearing tree that belongs to the Anacardiaceae family. It is a deciduous, erect tree that can grow up to 15-22 meters tall with a stem of 60-75 cm broad. The bark is grayish, marginally buttressed, and thick. The leaves of *S. mombin* are green, thin, and measure between 5-30 cm long. Most of the leaves are shed before the production of various small, white, fragrant flowers (Figure 6). The fruits of *S. mombin* are small, measuring between 2-4 cm long and 1.5-2 cm wide. They have a plum-like shape and are green in color. Upon ripening, the fruits turn yellow. The fruit has a thin, edible skin and a fibrous, juicy pulp that surrounds a hard, woody seed. *S. mombin* is commonly found in the lowland moist forest of the Amazon. It is an important source of food for many animals and is also used for medicinal purposes in traditional medicine [43]. The trunk is incised deeply in the bark, often producing a brown resin. *S. mombin* is not only found in the lowland moist forest of the Amazon, but it is also distributed throughout tropical America, Brazil, Nigeria, West Indies, and other tropical rainforests of the earth.

Figure 6: *Spondias mombin* tree along with leaves and fruits

The taxonomic hierarchy of *S. mombin*:

- **Kingdom:** Plantae (Plant)
- **Subkingdom:** Tracheobionta (Vascular Plants)
- **Superdivision:** Spermatophyta (Seed Plants)
- **Division:** Magnoliophyta (Flowering Plants)
- **Class:** Magnoliopsida (Dicotyledons)
- **Subclass:** Rosidae
- **Order:** Sapindales
- **Family:** Anacardiaceae

- **Genus:** Spondias
- **Species:** *Spondias mombin*

Ecological Descriptions of *S. Mombin:* *S. mombin*, commonly known as the hog plum, is a versatile plant species that can thrive in a variety of soil types and rainfall patterns, particularly in humid tropical climates. While the hog plum can tolerate a wide range of soil types and rainfall patterns, it is vulnerable to freezing stress and can suffer serious damage as a result. In drier regions, the plant can still be found, particularly in elevated fertile fields where the land is waterlogged for two or three months of the year. It occurs generally in the rainforest and coastal regions. It is available in almost all parts of Nigeria and needs a temperature not exceeding 25^{o} C. The size of the yield is virtually abundant [44].

Ethnomedicinal and Folkloric Uses of *S. mombin*

Spondias mombin is a plant that has been traditionally used in herbal medicine for several human diseases and ailments in tropical regions across the globe. The plant's leaves, fruits, bark, and stem have been used for various ethnomedicinal and folkloric purposes, including treating inflammatory disorders, infections, wounds, and several other human diseases [45]. Tables 1 and 2 summarize the ethnomedicinal and folkloric uses of *Spondias mombin* Linn. respectively.

Table 1: Ethnomedicinal Uses of Different Morphological Parts of *S. mombin*

Parts used	Medicinal Uses	Methods of Preparation
Root	Purgative	Decoction
Bark	As emetic, a remedy for diarrhea, dysentery, haemorrhoids, expel calcification from the bladder.	Decoction
Gums	Expectorant, and with the intention of expelling tapeworm	
Leaves	Stomach ache, biliousness, diarrhea inflammation, and dysentery.	Decoction
Fruit	Diuretic and antipyretic	Juice
Flowers	Inflammation of the eyes, the throat, and the bile ducts	Tea

Table 2: Folkloric Uses of *Spondias mombin* [46]

S/N	Morphological part	Non-Medicinal Uses
1	Root	It's an interim water supply in case of an emergency.
2	Stem	Agriculture with living fences. Shelter built by skilled craftspeople using a scarce resource.
3	Gum	Adhesive
4	Leaves	The tender new leaves are a popular culinary addition.
5	Fruit	This fruit can be eaten raw, stewed with sugar, or extracted to make ice cream, chilled beverages, or jam. In Panama, Peru, and Mexico, it is used as jam; in Guatemala, it is made into wine; and in Mexico, it is pickled to make vinegar. It has high market value as livestock and livestock feed.
6	Flowers	It's solely aesthetic.

Biological Activities of *Spondias mombin* Linn.

In traditional medicine, all components of the *S. mombin* tree are medicinally essential. Early studies indicate that *Spondias mombin* phenolic acid and 6-alkenyl-salicylic acid are accountable for this plant's antibacterial and molluscicidal properties [47]. In another research, it has been shown that the anacardic acid derived from the plant's hexane extract has inhibitory characteristics of beta-lactamase [48]. The results were based on novelty-induced behaviour, effects on sleep time initiated by hexobarbitone, swim tests, and induced seizure effects performed using conventional pharmacological instruments. *S. mombin* extracts have shown a variety of biological activities, which are summarised in Table 3.

Table 3: Extracts from the *S. mombin* Plant Illustrate Biological Activity

Biological Activity	References
Abortifacients	[49]
Anti-epileptic and Anti-psychotic	[50]
Anti-viral and Anti-bacterial	[51]
Anti-fertility	[52]
Antiinflammation	[53]
Anti-microbial	[54]
Reduced glutathione synthesis	[55]
Antitumour	[56]

Phytochemistry of *Spondias mombin* Linn.

Carotenoids, phytoene, α-trans- β -carotene, alpha-carotene, β -crytoxanthin (cis and trans), zeinoxanthin, and lutein were found in *S. mombin* seeds by high performance liquid chromatography (HPLC) analysis, as reported by Hamano *et al.* (2001) [57]. The seed has 31.5 % oil content. Approximately half of the weight of the fruit is pulp (8 %) water, sugar (10 %), fibre (1 – 8 %), and ash (0.4 %). The sugars yield about 40 calories/100 g. The fruit of *S. mombin* is also a rich source of vitamin C, with concentrations of up to 70 mg per 100 g of fresh fruit. The seeds have been found to contain significant amounts of fatty acids, with oleic acid being the most abundant [58]. One of the plant's traditional medicinal uses—memory enhancement—is supported by the discovery of cholinesterase inhibitory chemicals extracted from the plant.

Conclusion:

The review highlights that *Spondias mombin* exhibits the ability to regulate various molecular pathways involved in cancer development and progression. These pathways include IAPs (Inhibitor of Apoptosis Proteins), XIAP (X-linked Inhibitor of Apoptosis Protein), TNF-α (Tumor Necrosis Factor-alpha), IL-1β (Interleukin-1beta), and KIM-1 (Kidney Injury Molecule-1). By modulating these molecular pathways, *Spondias mombin* has the potential to exert anti-cancer effects. IAPs and XIAP are proteins that play a role in inhibiting apoptosis, or programmed cell death, which is a mechanism that can eliminate cancer cells. *Spondias mombin* may inhibit these proteins, promoting apoptosis and inhibiting cancer cell survival. Moreover, *S. mombin* may influence TNF-α and IL-1β, which are pro-inflammatory cytokines associated with cancer progression. By regulating these cytokines, *S. mombin* may help control inflammation and suppress tumor growth. Overall, the review indicates that *S. mombin* possesses ethnobotanical properties that can potentially be harnessed for cancer management. By regulating various

molecular pathways, including IAPs, XIAP, TNF-α, IL-1β, and KIM-1, *S. mombin* shows promise as a natural compound with anti-cancer effects. However, further research, including in vivo and clinical studies, is needed to validate these findings and explore the full potential of *S. mombin* in cancer treatment and prevention.

References:

1. Shyur, L.F., 2012. *Recent trends in medicinal plants research*. Academic Press.
2. Balogun, J.A., 2022. Emerging Developments in Traditional Medicine Practice in Nigeria. In *The Nigerian Healthcare System: Pathway to Universal and High-Quality Health Care* (pp. 235-275). Cham: Springer International Publishing.
3. Eshete, M.A. and Molla, E.L., 2021. Cultural significance of medicinal plants in healing human ailments among Guji semi-pastoralist people, Suro Barguda District, Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, *17*(1), pp.1-18.
4. Cordero, C.S., Meve, U. and Alejandro, G.J.D., 2022. Ethnobotanical documentation of medicinal plants used by the indigenous panay bukidnon in lambunao, iloilo, Philippines. *Frontiers in Pharmacology*, p.3555.
5. Sundaram, M., Guernsey, D.L., Rajaraman, M.M. and Rajaraman, R., 2004. Neosis: a novel type of cell division in cancer. *Cancer biology & therapy*, *3*(2), pp.207-218.
6. Adebamowo, C.A. and Adekunle, O.O., 1999. Case-controlled study of the epidemiological risk factors for breast cancer in Nigeria. *British Journal of Surgery*, *86*(5), pp.665-668.
7. Parkes, C.M., 1972. Accuracy of predictions of survival in later stages of cancer. *Br Med J*, *2*(5804), pp.29-31.
8. Marx, J.L., 1989. How Cancer Cells Spread in the Body: Metastatic cancer cells need to undergo several changes in order to make the arduous journey from the primary tumor where they originated to new target sites. *Science*, *244*(4901), pp.147-148.
9. Gatto, N.M., 2021. Environmental carcinogens and cancer risk. *Cancers*, *13*(4), p.622.
10. Gallagher, E.J. and LeRoith, D., 2020. Hyperinsulinaemia in cancer. *Nature Reviews Cancer*, *20*(11), pp.629-644.
11. Zhou, W., Ding, Q., Pan, H., Wu, N., Liang, M., Huang, Y., Chen, L., Zha, X., Liu, X. and Wang, S., 2014. Risk of breast cancer and family history of other cancers in first-degree relatives in Chinese women: a case control study. *BMC cancer*, *14*, pp.1-7.
12. Michaud, J.E., Billups, K.L. and Partin, A.W., 2015. Testosterone and prostate cancer: an evidence-based review of pathogenesis and oncologic risk. *Therapeutic advances in urology*, *7*(6), pp.378-387.
13. Patel, A., 2020. Benign vs malignant tumors. *JAMA oncology*, *6*(9), pp.1488-1488.
14.] Dendy, P.P. and Meldrum, R.A., 1979. CHARACTERISTICS OF THE MALIGNANT CELL AND THE CELL CYCLE IN TUMOURS. In *Placenta* (pp. 176-214). Pergamon.
15. Kaczanowski, S., 2016. Apoptosis: its origin, history, maintenance and the medical implications for cancer and aging. *Physical Biology*, *13*(3), p.031001.
16. Wong, R.S., 2011. Apoptosis in cancer: from pathogenesis to treatment. *Journal of experimental & clinical cancer research*, *30*(1), pp.1-14.

17. Riedl, S.J. and Shi, Y., 2004. Molecular mechanisms of caspase regulation during apoptosis. *Nature reviews Molecular cell biology*, *5*(11), pp.897-907.
18. Kumar, R., Herbert, P.E. and Warrens, A.N., 2005. An introduction to death receptors in apoptosis. *International Journal of Surgery*, *3*(4), pp.268-277.
19. Yang, J.K., 2015. Death effecter domain for the assembly of death-inducing signaling complex. *Apoptosis*, *20*, pp.235-239.
20. Tsujimoto, Y., 1998. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?. *Genes to cells*, *3*(11), pp.697-707.
21. Qian, S., Wei, Z., Yang, W., Huang, J., Yang, Y. and Wang, J., 2022. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Frontiers in Oncology*, *12*.
22. Fischer, U. and Schulze-Osthoff, K., 2005. Apoptosis-based therapies and drug targets. *Cell Death & Differentiation*, *12*(1), pp.942-961.
23. Carneiro, B.A. and El-Deiry, W.S., 2020. Targeting apoptosis in cancer therapy. *Nature reviews Clinical oncology*, *17*(7), pp.395-417.
24. Chota, A., George, B.P. and Abrahamse, H., 2021. Interactions of multidomain pro-apoptotic and anti-apoptotic proteins in cancer cell death. *Oncotarget*, *12*(16), p.1615.
25. Devarajan, E., Sahin, A.A., Chen, J.S., Krishnamurthy, R.R., Aggarwal, N., Brun, A.M., Sapino, A., Zhang, F., Sharma, D., Yang, X.H. and Tora, A.D., 2002. Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. *Oncogene*, *21*(57), pp.8843-8851.
26. Fulda, S., 2011. Targeting apoptosis signaling pathways for anticancer therapy. *Frontiers in oncology*, *1*, p.23.
27. Berthelet, J. and Dubrez, L., 2013. Regulation of apoptosis by inhibitors of apoptosis (IAPs). *Cells*, *2*(1), pp.163-187.
28. Oberoi-Khanuja, T.K., Murali, A. and Rajalingam, K., 2013. IAPs on the move: role of inhibitors of apoptosis proteins in cell migration. *Cell death & disease*, *4*(9), pp.e784-e784.
29. Tu, H. and Costa, M., 2020. XIAP's profile in human cancer. *Biomolecules*, *10*(11), p.1493.
30. Hussain, A.R., Siraj, A.K., Ahmed, M., Bu, R., Pratheeshkumar, P., Alrashed, A.M., Qadri, Z., Ajarim, D., Al-Dayel, F., Beg, S. and Al-Kuraya, K.S., 2017. XIAP over-expression is an independent poor prognostic marker in Middle Eastern breast cancer and can be targeted to induce efficient apoptosis. *BMC cancer*, *17*(1), pp.1-13.
31. Abbas, R. and Larisch, S., 2020. Targeting XIAP for promoting cancer cell death—the story of ARTS and SMAC. *Cells*, *9*(3), p.663.
32. Yang, L., Li, A., Lei, Q. and Zhang, Y., 2019. Tumor-intrinsic signaling pathways: key roles in the regulation of the immunosuppressive tumor microenvironment. *Journal of hematology & oncology*, *12*(1), pp.1-14.
33. Grivennikov, S.I., Greten, F.R. and Karin, M., 2010. Immunity, inflammation, and cancer. *Cell*, *140*(6), pp.883-899.
34. Zhao, H., Wu, L., Yan, G., Chen, Y., Zhou, M., Wu, Y. and Li, Y., 2021. Inflammation and tumor progression: signaling pathways and targeted intervention. *Signal transduction and targeted therapy*, *6*(1), p.263.

35. Josephs, S.F., Ichim, T.E., Prince, S.M., Kesari, S., Marincola, F.M., Escobedo, A.R. and Jafri, A., 2018. Unleashing endogenous TNF-alpha as a cancer immunotherapeutic. *Journal of translational medicine*, *16*, pp.1-8.
36. Martínez-Reza, I., Díaz, L. and García-Becerra, R., 2017. Preclinical and clinical aspects of TNF-α and its receptors TNFR1 and TNFR2 in breast cancer. *Journal of Biomedical Science*, *24*(1), pp.1-8.
37. Mercogliano, M.F., Bruni, S., Elizalde, P.V. and Schillaci, R., 2020. Tumor necrosis factor α blockade: an opportunity to tackle breast cancer. *Frontiers in oncology*, *10*, p.584.
38. Oh, K., Lee, O.Y., Park, Y., Seo, M.W. and Lee, D.S., 2016. IL-1β induces IL-6 production and increases invasiveness and estrogen-independent growth in a TG2-dependent manner in human breast cancer cells. *Bmc Cancer*, *16*, pp.1-11.
39. Baker, K.J., Houston, A. and Brint, E., 2019. IL-1 family members in cancer; two sides to every story. *Frontiers in immunology*, *10*, p.1197.
40. Bonventre, J.V., 2014. Kidney injury molecule-1: a translational journey. *Transactions of the American Clinical and Climatological Association*, *125*, p.293.
41. Kim, E.K. and Choi, E.J., 2010. Pathological roles of MAPK signaling pathways in human diseases. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1802*(4), pp.396-405.
42. Zhang, Y. and Wang, X., 2020. Targeting the Wnt/β-catenin signaling pathway in cancer. *Journal of hematology & oncology*, *13*, pp.1-16.
43. Tiburski, J.H., Rosenthal, A., Deliza, R., de Oliveira Godoy, R.L. and Pacheco, S., 2011. Nutritional properties of yellow mombin (Spondias mombin L.) pulp. *Food Research International*, *44*(7), pp.2326-2331.
44. Ayoka, A.O., Akomolafe, R.O., Akinsomisoye, O.S. and Ukponmwan, O.E., 2008. Medicinal and economic value of Spondias mombin. *African Journal of Biomedical Research*, *11*(2).
45. Njoku, P.C. and Akumefula, M.I., 2007. Phytochemical and nutrient evaluation of Spondias mombin leaves. *Pak. J. Nutr*, *6*(6), pp.613-615.
46. Adedokun, M.O., Oladoye, A.O., Oluwalana, S.A. and Mendie, I.I., 2010. Socio–economic importance and utilization of Spondias mombin in Nigeria. *Asian Pacific Journal of Tropical Medicine*, *3*(3), pp.232-234.
47. Ogunro, O.B., Oyeyinka, B.O., Gyebi, G.A. and Batiha, G.E.S., 2023. Nutritional benefits, ethnomedicinal uses, phytochemistry, pharmacological properties and toxicity of Spondias mombin Linn: a comprehensive review. *Journal of Pharmacy and Pharmacology*.
48. Coates, N.J., Gilpin, M.L., Gwynn, M.N., Lewis, D.E., Milner, P.H., Spear, S.R. and Tyler, J.W., 1994. SB-202742, a novel β-lactamase inhibitor isolated from Spondias mombin. *Journal of natural products*, *57*(5), pp.654-657.
49. Offiah, V.N. and Anyanwu, I.I., 1989. Abortifacient activity of an aqueous extract of Spondias mombin leaves. *Journal of ethnopharmacology*, *26*(3), pp.317-320.
50. Ayoka, A.O., Akomolafe, R.O., Iwalewa, E.O., Akanmu, M.A. and Ukponmwan, O.E., 2006. Sedative, antiepileptic and antipsychotic effects of Spondias mombin L.(Anacardiaceae) in mice and rats. *Journal of Ethnopharmacology*, *103*(2), pp.166-175.

51. Cabral, B., Siqueira, E., Bitencourt, M.A., Lima, M.C., Lima, A.K., Ortmann, C.F., Chaves, V.C., Fernandes-Pedrosa, M.F., Rocha, H.A., Scortecci, K.C. and Reginatto, F.H., 2016. Phytochemical study and anti-inflammatory and antioxidant potential of Spondias mombin leaves. *Revista Brasileira de Farmacognosia*, *26*, pp.304-311.
52. Asuquo, O.R., Ekanem, T.B., Udoh, P.B. and Eluwa, M.A., 2012. Histomorphological study of the anti-fertility effect of Spondias mombin L. in adult male rats. *IOSR J Pharm Biol Sci*, *3*(2), pp.29-34.
53. Nworu, C.S., Akah, P.A., Okoye, F.B., Toukam, D.K., Udeh, J. and Esimone, C.O., 2011. The leaf extract of Spondias mombin L. displays an anti-inflammatory effect and suppresses inducible formation of tumor necrosis factor-α and nitric oxide (NO). *Journal of immunotoxicology*, *8*(1), pp.10-16.
54. de Lima, I.P.A., Alves, R.A.H., Mayer, J.D.S.L., Costa, M.R.M., de Mendonça, A.K.P., de Lima, E.L.F., Langassner, S.M.Z., Dametto, F.R., de Lima, K.C. and Lins, R.D.A.U., 2021. Antimicrobial activity of Spondias mombin L. aqueous and hydroethanolic extracts on Enterococcus faecalis and *Pseudomonas aeruginosa*-an in vitro study. *Research, Society and Development*, *10*(1), pp.e50710111949-e50710111949.
55. Igwe, C.U., Onyeze, G.O.C., Onwuliri, V.A., Osuagwu, C.G. and Ojiako, A.O., 2010. Evaluation of the chemical compositions of the leaf of Spondias mombin Linn from Nigeria. *Australian Journal of Basic and Applied Sciences*, *4*(5), pp.706-710.
56. Boadu, A., Karpoormath, R. and Nlooto, M., 2022. Ethnomedicinal, Phytochemistry and Pharmacological Actions of Leaf Extracts of Spondias Mombin: A Narrative Review. *African Journal of Biomedical Research*, *25*(1), pp.1-11.
57. Tiburski, J.H., Rosenthal, A., Deliza, R., de Oliveira Godoy, R.L. and Pacheco, S., 2011. Nutritional properties of yellow mombin (Spondias mombin L.) pulp. *Food Research International*, *44*(7), pp.2326-2331.
58. Ogunro, O.B., Oyeyinka, B.O., Gyebi, G.A. and Batiha, G.E.S., 2023. Nutritional benefits, ethnomedicinal uses, phytochemistry, pharmacological properties and toxicity of Spondias mombin Linn: a comprehensive review. *Journal of Pharmacy and Pharmacology*.

Research and Development in Pharmaceutical Science Volume V

(ISBN: 978-93-88901-53-6)

About Editors

Mr. Ajit Kumar Varma has obtained his B.Pharm from Gautam Buddh Technical University(GBTU), Lucknow,And M.Pharm from Rajiv Gandhi Proudyogiki Vishwavidyalaya (RGPV),Bhopal.He is currently working as an Asst.Professor at Faculty of Pharmaceutical Sciences,Rama University,Kanpur (U.P.),India. He has above Seven years of Pharmaceutical Industry and teaching in good experience. He has attened several National and International Conferences/Workshops Seminars. He has credit in Nobel Researchers Awards as well as Young Scientist Awards and published several research paper in International Journals, conferences and proceedings.He has been Reviewer of several Journals as well as different Membership of Educational Society .

Dr. M. Shanthala obtained her Master Degree from Kuvempu University, Shimoga in the year 2001, awarded Ph.D degree in 2006. She has published scientific papers in reputed National and International journals including book articles and chapters. Basically, trained meticulously in the Cytological and Biochemical techniques, having more than 15 years of PG teaching and research experience at the Centre for Applied Genetics, Bangalore University, Bengaluru. She has more than 200 Citations. Her area of research includes Aquatic Biology and Toxicology.

Dr. Pratibha has completed her Bachelor's degree in Agriculture from BAU, Sabour, Bihar and Master's degree in Genetics and Plant Breeding from IGKV, Raipur, Chhattisgarh. She has obtained her Ph.D. degree in Genetics and Plant Breeding from AAU, Anand, Gujarat. She has successfully passed many prestigious exams such as ICAR-JRF, ICAR-SRF, ICAR-NET (ASRB) and received awards such as 'Best Ph.D. thesis' award and 'Young professional' award. She has also published several research papers, review papers and book chapters in various journals and publications of national and international repute.

Dr. Pralhad Rege, working in capacity of Assistant Professor in the Department of Chemistry, St. Xavier's College Mumbai. He has completed his PhD in Analytical Chemistry. He hold PGDHRM from Symbiosis, Pune and PG Diploma in Clinico-Regulatory and Patents from Ruia College. His total teaching experience is 14 years and research experience is 12 years. He is Mumbai University recognized guide for M.Sc by research and PhD. Currently two students for M.Sc by research and three students for PhD are working under his guidance. His area of research interest is Analytical chemistry, Pharmaceutical chemistry, synthetic chemistry and Herbal science. He has published 22 research papers in various national and international, actively participated in more than 70 conferences / workshops/ Seminars/ online FDP's. Till date he has Presented 15 research papers in the form of Oral / poster presentations. He has received Minor Research Grant from University of Mumbai for the Academic year 2016-17. He has Worked as Reviewer for the book. He has Written a book titled "Concise Graduate Chemistry –III published by University of Mumbai. He is a visiting faculty in Ruia, K.J.Somaiya, Mithibai, Guru Nanak, Vivekanand and Konkan Gyanpeeth, Karjat College. He has invited as a Recourse Person in Chemistry in the International Junior Science Olympiad-Cum-Selection Camp (OCC) at K.J.Somaiya College. He is a recipient of Best Researcher Award by VDGOOD Professional Association Chennai, Tamil Nadu, India.

Bhumi Publishing

ISBN 978-93-88901-53-6

9 789388 901536