PHOSPHOLIPASE A_2 INHIBITORY ACTIVITY OF TRIPHALA PLANTS CONSTITUENTS FOR THEIR APPLICATIONS IN THE TREATMENT OF INFLAMMATORY AND ONCOLOGY DISEASES

A Thesis

Submitted in partial fulfillment of the requirements for the

Award of the degree of

DOCTOR OF PHILOSOPHY

In

(BOTANY)

By

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PUNJAB

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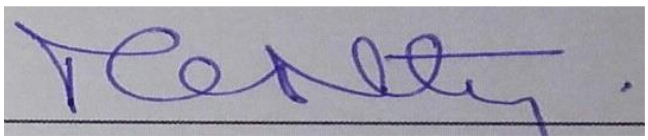
DECLARATION

I hereby declare that the thesis entitled, **"Phospholipase A_2 Inhibitory Activity of Triphala Plants Constituents for Their Applications in The Treatment of Inflammatory and Oncology Diseases"** submitted for Degree of Ph.D. in Botany to Department of Botany, Lovely Professional University. This is original research work done by me, the ideas and references were acknowledged. This research idea and the work was not used for awarding any degree.



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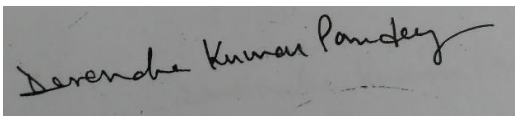

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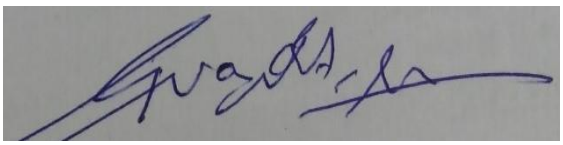

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ABSTRACT

The enzyme which can efficiently control the inflammations, their relative diseases such as carcinoma is Secretory PLA_2 ($sPLA_2$). $sPLA_2$ used in treating the organism affected by the carcinoma. $sPLA_2$ is found more in bio enzymes means, the enzymes collected from plants. In ancient times our forefathers were using plant extracts for curing various kinds of health ailments as they don't have any side effects. In the olden days, diseases were cured with plant extracts, through which now we learned to extract different drugs to prepare biomedicines. Triphalachuran is extracted from three different plants like *Phyllanthus emblica, Terminalia chebula, and Terminalia bellirica.* From the fruits of these plants, we make Triphalachuran, which is used widely in Ayurvedic medicines. Triphalachuran is having many types of flavonoids, alkaloids, terpenoids and glycosidic compounds. Triphala powder has excellent capacity in controlling activities of $sPLA_2$ and acts as anti-carcinoma. These activities were studied under special conditions and based on the values,it is easy to get *insilico* activities. Triphalachuran contains $sPLA_2$ controlling agents and hence is an anti-carcinogenic agent. The antioxidant nature of the extract is identified with the help of DPPH, dot-blot assay and hydrogen peroxide assay. The specific feature of Morin (3,5,7,2,5-pentahydroxy flavone) and 9, 10 anthraquinone, which are the compounds of Triphalachuran, are toxic to breast cancer cells. They have the ability to control the rapid increase of infected cells in breasts. When treated with Morin and 9, 10 anthraquinone, it is found that 85% of cyst is decreased in MDA MB-231 Cell lines. This is found with the help of MTT assay. Cell proliferation is controlled by Morin and 9, 10 anthraquinone could be measured with thymidine analogBrdU (5 bromo-2'- deoxyurodome) as it is incorporated for subsequent detection.

Cell death occurs by treating human Breast Cancer cells (MDA-MB-231) with ethidium bromide. After 24hrs, through Flow cytometry, we can see the rise in G0/G1 phase in breast cancer cell numbers and decrease in S and G2 phase ($P<0.05$). By seeing the above results, we can use Morin and Anthraquinone as cancer controlling agent as it kills the breast cancer cells and stops them from making apoptosis.

Keywords: Triphalachuran, Docking, Breast cancer cell lines, anticancer compounds, Morin and Anthraquinone.

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Satyanarayana Murthy Malladi

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%	Percentage
^{0}	Degree
^{0}C	Degree Celsius
^{0}F	Degree Fahrenheit
<	Less than
>	Greater than
µ	Micro
µl	Microliter
µM	Micromolar
AA	Arachidonic acid
ADME	Absorption, distribution, metabolism, and excretion
AdPLA	Adipose-specific PLA_2
ARDS	Acute respiratory distress syndrome
BAIC	Bioactive immunomodulatory compound
BSI	Botanical Survey of India
CAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
DISC	Death-inducing signal complex
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl.
EDTA	Ethylenediamine tetra acetic acid
FADD	Fas-associated death domain protein
FBS	Fetal bovine serum

FDA	Food and Drug Administration
GC_MS	Gas chromatography–mass spectrometry
H2O2	Hydrogen peroxide
HIF	Hypoxia-inducible factor
HIF-1a	Hypoxia-inducible factor 1-alpha
HPLC	High-performance liquid chromatography
IARC	The International Agency for Research on Cancer
LDL	Low-density lipoprotein
LLC	Lewis lung carcinoma
$LpPLA_2$	Lipoprotein-associated phospholipase A_2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEC	Neonatal necrotizing enterocolitis
NEL	Neonatal necrotizing enterocolitis
NF-KB	Nuclear factor kappa light chain enhancer of activated B cells
NIST	National Institute of Standards and Technology
NO2	Nitrogen dioxide
PAF	Platelet-activating factor
PC	Phosphatidylethanolamine
PE	Phosphatidylcholine
PLA_2	Phospholipase A_2
PMSF	Phenylmethylsulphonyl fluoride
ROS	Reactive Oxygen Species
TLC	Thin-layer chromatography
WHO	World Health Organization

CHAPTER - 1

INTRODUCTION

1.1 History of the Ancient Medicinal plants:

The Herbal medicinal system was the oldest medicinal system from ancient times. In the present-day system (Modern system) also, this medicinal system is adopted in their regular lifestyle. The Ancient medicinal system entirely depends on medicinal plants for different health issues **(**Fabricant and Francisworth *et al.,* 2001). Even today, the maximum number of people turn to the herbal medicinal system and products of the medicinal plants, which are available from Primary Health Care Centres, to compensate for their needs (Rawant and Goyal *et al.,* 2009). From 500-300 BC onwards, human beings started vigorous usage of medicinal plant extracts and plant parts tomaintain good health. The therapeutic usage of them started first in Mesopotamia in 2600 BC; they used to extract different oils from different plants like cypress, liquorice, cedar, poppy and myrrh for various problems and different infections too. From the Mesopotamian civilisation to the present generations, some plants are under continuous usage (Ramawat *et al.,* 2007). They are *Glycyrrhiza glabra*, *Papaver somniferum*, *Cupressus sempervirens*, *Commiphorawightii* and many other plants.

The type of treatment, native to a particular region, culture or country and practised traditionally in that region, is called a Traditional system of Medicine or Indigenous Medicine or Ethnomedicine. The traditional method of medicine is defined as "Sum of the skills, knowledge and practices based on believes, theories and experiences, indigenous to different cultures used in the maintenance of health, identifying, preventing or treating physical and mental illness". Because of the belief and treating so many diseases by the Traditional system of medicine, it leads to inventing new drugs for preventing and treating human health. So, the bioactive substances of the plants have shown their importance in traditional medicine, food supplements, nutraceuticals and pharmaceutical industries (Ncube *et al.,* 2008). As WHO analysis and reports still, more than 75% of people in the world use plant extracts or

traditionally known treatment for curing health-related issues. (Winston *et al.*, 1999)For different problems, extensive use of herbal drugs is increased because of not having side effects and easy availability, including the industrially developed countries. This traditional system of medicine is used with different names in different places and different countries, as Ayurveda (India), Acupuncture (China), Unani (Arab countries), Naturopathy and Yoga in Kerala (India).

1.2 Indian Traditional Medicine:

In the world, India places a different position because of having many traditional systems of medicine. All these were practised regularly in the sub-continent of India to maintain health as Primary Health Care. Ayurveda, Siddha, Unani, Homoeopathy and Naturopathy come under the traditional systems of medicine. Out of all these, the oldest and most accepted medicinal system in India is Ayurveda. All other medicinal systems came based on this system alone. The word "Ayurveda" came from "Ayuh" (means Life) and "Veda" (means science or knowledge). So, Ayurveda means 'Science of Life'. The Indo-Aryans mentioned the medicinal plants in our Vedas. The world's oldest religious book –"Rigveda" (Kirikar and Basu *et al.*, 1958), in this, we mentioned the medicinal plant 'Soma'. By 200 BC in Rigveda and Atharvana Veda, they referred to the different diseases and their treatment with plant extracts. We can say that Ayurveda came from Athervana Veda, having 6599 hymns and 700 lines (Prose). Aryans mentioned ancient medicinal therapy in Susrutha Samhita and Charaka Samhita, the medicinal plants and their therapeutic usages (Steiner *et al.*, 1986). Sushruta Samhita (600 BC) and Charaka Samhita (900 BC) mentioned about nearly 700 medicinal plants uses and their therapeutic usage (Singh and Abrar *et al.*, 1990). We have invented the most useful medicines for malaria, cardiac problems and several others from the plants' Quassia, Physiostigma, Digitalis, and Cinchona from a traditional system of medicines. All this information was written clearly in these books regarding how to extract medicines from the different plant parts for different health Issues.

The Ayurvedic theories depend on the concept of 'Panchamahabhutas' and 'Tridoshas' Ayurveda says that five major elements together build a human being. They are Akash, Vayu, Agni, Jal and Prithvi. These elements are omnipresent (widespread), in relative proportion and interacting with each other makes the world going. Space denotes spaces with in body like mouth, nostrils and abdomen; Air indicates the muscular movement, Fire controls the enzyme functions (intelligence, digestion & other metabolic activities). All sorts of body fluids are made up of water like saliva, plasma in blood etc. Earth refers to the structure of humans. The body is made of bones, muscles are made with flesh; for digestion, teeth andmanyothersare the references. All digestive and treatment procedures depend on thesepanchamahabhutas Ayurveda. The increase and advancement of the body depend upon the composition and nutrients of the food. The composition of the panchamahabhutas in food also nourishes the body and tissues.

Tridoshas –all the five panchamahabhutas in the body represent three doshas(Physical energies). They are Vata, Pitta and Kapha. These tridoshas govern the entire body functions and physical structure. Even they make the body grow and decay. Space and air come together to form Vata. Vata checked all the activities and functions of the body. When vata intensifies in the body, it shows constipation, insomnia, sensory disorientation, physical weakness; fire and water together make pitta, which involves the digestion of food. It can control digestion, heat, hunger, thirst, lustre, intelligence, the softness of the body. Pitta is acid present in the intestine, stomach, blood, lymph, visual organelles. Pitta presents primarily in the small intestine. When it is more in the body than the urine, stool colour changes to yellow. When the levels increase drastically, then the skin and eye colour change to yellow. Water and earth combine to form Kapha (phlegm). Kapha gives strength and stability to the body by maintaining the internal environment. Kapha is responsible for emotional feelings such as love, patience, compassion and forgiveness. Kapha is a force, which causes mucus to arise. Due to the increased amount of Kapha in our body, we experience chill, heaviness, loosening of limbs, cough, excessive sleeping and difficulty in breathing.

1.3 Chinese Traditional Medicine:

In China, for more than 200 years, only one primary traditional system is in practice, i.e., Traditional Chinese Medicine. Before the use of Allopathy in China, most of the health problems were cured by Chinese herbal products. "Yellow Emperors Inner Classic" written by Hang DiNei Jing (200 BC to 100 AD), "Divine Husband Man's Classic of Material Medica" by Shen Nong Ben Cao Jing (25 to 220 AD) and "Cold Induced Disorders" by Shang Han Lun (220 AD) are the oldest books on Chinese Medicine. The book published in 1977 is 'Chinese Materia Medica' includes nearly 6000 drugs. The plants used in Traditional Chinese Medicine are Seagrape, Radix angelica, Common garden peony *Rheumpalmatum*, *Urena lobata* Artemisia annuna, Panax ginseng **(**Zhang & Zia *et al.*, 2007).

1.4 Traditionally used Egyptian medicine:

Traditional medicine used by Egyptians was there since 3000 BC. Papyrus Ebers wrote the ancient medicinal literature record in 1500 BC, mentions some medicinal plant seeds of *Ricinus communis, Citirilus colocynth, Senna alexandrina roots, and Punica granatum*. After 100 AD, Dioscorides, a Greek physician, mentioned this medicinal plant uses again. By 650 BC Assyrian & Babylonian medicinal knowledge emerged. Greeks developed the world's first 'Materia Medica'. The Greek physician Hippocrates (460 BC), known as the 'Father of Medicine', established the first pharmacy in the world. He described nearly 400 medicinal plants and mentioned their names too. Theophrastus (370 – 267 BC), known as 'Father of Botany', mentioned about 500 medicinal plants in his book "de Historia Plantarum". The roots of the Modern Medicinal system (Allopathy) is in Egyptian civilisation (Heinrich *et al*., 2004).

1.5 Arabic traditional medicine:

The persons who precede their culture had a proper knowledge of the medicinal plants and their usage at 632 – 1150 AD, considered as the Golden age period of Muslims. Arabs established drug stores in the eighthcentury itself. In their Prophet

Muhammad (SAW) teachings and Holy book "Quran", mentioned personal hygiene. In books of Quran and Hadith mentioned the different medicinal plants and their usage. Having a better knowledge of the medicinal plants, Prophet Mohammad (SAW) and his followers treated many people with various problems (Guorra *et al.*, 1979). The plants used for treating diseases by Arabs used different plants *like Papaver somniferum, Tragacanth, Onion, Ferula asafoetida, Carthamus tinctorius, Carum carvi, Peganum harmala, Prunus dulcis, Lawsoniainermis, Punica granatum, Mustard plant, Sesame, Indian sennaTrigonella foenumgraecum, Olea europaea, Vitis vinifera and Trachyspermumammi.*

This traditional knowledge about medicinal plants and their usage opens the gates for further investigations in drug discovery. After inventing so many drugs, still, researchers believe that plants are having many more reserves of chemical compositions to treat the human body. This knowledge makes us understand medicinal plants and traditional usages.

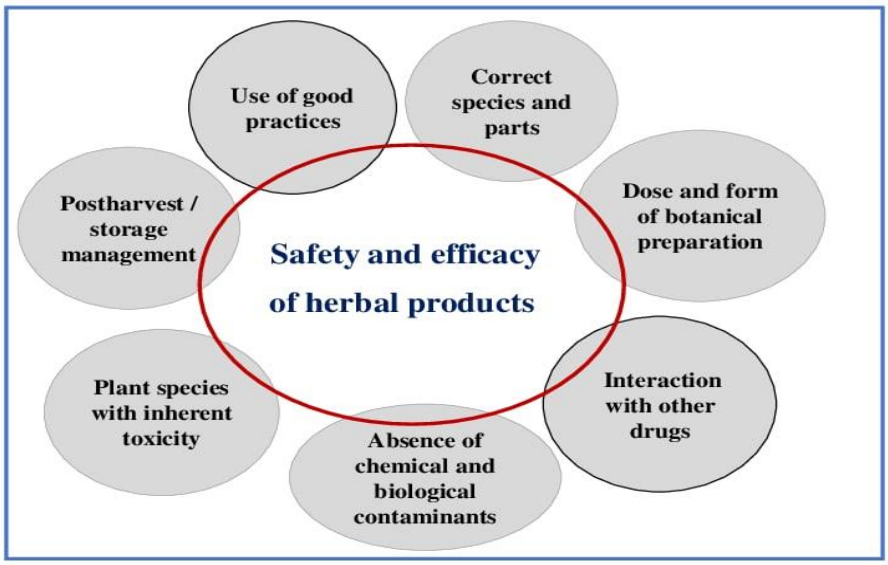
1.6 Natural Plant products in Modern Medicine

Natural chemical products are going to obtain from plants and other living organisms. The study of Natural chemical products means to study from which part we have extracted, their molecular formula, chemical structure, biological function, application in the organisms and also their uses. These chemical products are the secondary metabolites in the plants used in different ways. This makes the plants in surviving in extreme climatic conditions or protect them from grazing animals or as a defence mechanism (Calixto *et al.,* 1996). The secondary metabolites are useful in making drugs, healthy food or the right supplements of growth.

Plants are the primary sources for extracting bioactive compounds. In the US and European countries, half of the drug composition is derived from plants only. (Cordell *et al.,* 2002). The estimated number of different plans on earth is 2,50,000 to 5,00,000. In that total number of plants, phytochemical plants are only 15% and less

than 6% of plants are biologically active (Verpoorte *et al.*, 2000). More plants are yet to be studied to get natural alkaloids or drugs (Cragg & Newman *et al.*, 2005).

So many scientists studied and invented many therapeutic uses from thousands of aromatic plants and alkaloid yielding plants. They gave those drug isolation and applications for different diseases. From 100 medicinal plant species, they isolated 125 clinically proven useful drugs. Now, we need to cultivate those plants to get more drugs which are helpful for pharmaceutical companies.



Source: (Sabins and Daniel *et at* 1990; Dabuek *et al* 1991)

Fig. 1 Safety and Efficacy of Herbal Products

Table. 1 Shows some important medicinal plants whose bioactive molecules or alkaloids are used in Modern Medicine.

Drug	Source	Biological Activity
Allicin	*Allium sativum*	Hypolipidemic
Andrographolide	*Andrographis paniculata*	Hepatoprotective
Artemisinin	*Artemisia annua*	Antimalarial
Ajmalicine	*Rauwolfia canesocence*	Hypotensive
Atropine	*Solanum*	Anticholinergic
Bacoside	*Bacopa monneri*	Nootropic

Berberine	*Berberis lyceum*	Antiemetic
Boswellic acid	*Boswellia seratta*	Anti-inflammatory
Caffeine	*Camellia sinensis*	CNS stimulant
Camphor	*Cinnamomum camphora*	Aromatic
Capsaicin	*Capsicum annum*	Counter irritant
Cocaine	*Erythroxylum coca*	Analgesic
Codeine	*Papaver somniferum*	Anaesthetic
Colchicine	*Colchicum luteum*	Anti-inflammatory
Curcumin	*Curcuma longa*	Colouring pigment, Antioxidant
Digitoxin	*Digitalis lanata*	Cardiotonic
Disogenin/Stigmasterol	*Dioscoreadeltoidea*	Base material for steroids
Embelin	*Embeliaribes*	Anthelmintic
Emetine	*Cephaelis ipecacuanha*	Antiamoebic
Ephedrine	*Ephedraeherba*	Hypertensive
Ergotamine	*Claviceps purpurea*	Post Partumhaemorrhage
Etoposide/ tenopside	*Podophyllum emodii*	Anticancer
Forskolin	*Coleus forskolin*	Cardiotonic
Galanthamine	*Leucojum aestivum*	Anticholinesterase
Glycyrrhizin	*Glycyrrhiza glabra*	Antiviral
Gossypol	*Gossypium herbaceum*	Contraceptive
Guggulsterones/Gugallipid	*Commiphorawightti*	Hypocholeromic, Antiinflammatory
Hydroxy citric acid	*Garcinia cambogia*	Antiobesity agent
Hyoscine /Hyoscyamine	*Hyoscyamus niger, H.miticus, Datura metal*	Parasympathetic
Hypericin	*Hypericum perforatum*	Anti-HIV
Ipecac	*Cephaelis angustifolia*	Emetic

Lycopene	*Lycopersicon esculentum*	Antioxidant
Methoxsalen	*Ammi majus/ Heracleum candicans*	Leucoderma
Morphine/ papaverine	*Papaver somniferum*	Sedative
Podophyllotoxin	*Podophyllum hexandrum*	Anticancer
Psoralen	*Psoralea corylifolia*	Antileucoderma, Antibacterial
Quinine/Quinidine	*Cinchona officinalis*	Antimalarial
Reserpine	*Rauwolfia serpentine*	Hypotensive,
Rutin	*Sophora japonica/ Fagopyrum esculentum*	Vitamin-P
Sennoside	*Cassia angustifolia*	Laxative
Silymarin	*Silybium marianum*	Hepatoprotective
Strychnine	*Strychnosenux-vomica*	Central stimulant
Taxol	*Taxus wallichiana*	Anticancer
Tubocurarine	*Chondodendrontomentosum*	Muscle relaxant
Vasacine	*Adhatodavasica*	Vasodilatory
Vinblastine/ Vincristine	*Cathranthus roseus*	Anticancer
Valepotriates	*Valerianawallichii*	Sedative tranquillizer

After inventing new chemical drugs based on the natural drug formula, the natural drugs produced from medicinal plants are the primary source to control different new health problems (Raskin *et al.,* 2002). Despite chemical drugs, the demand for natural drugs was increasing nowadays. The increase in demand for natural medicines is because of their availability, cost, not having adverse effects and more effectiveness. (Eloff *et al.,* 1998; Ghosh *et al.,* 2008).

1.7 Medicinal plants – The present scenario:

According to Exim bank estimations, in the existing World business, only medicinal plants turnover is about 60 Billion US dollars. In that Rs.2300 crores from India's

Annual turnover (Sharma *et al.*, 2005), is with a growth rate of 7% per annum. Some countries that lead the Global Trade Center for MAPs are Germany, Italy, France, Japan, China, Spain, UK and the USA (Laird *et al.*, 1999). The demand for growing them is increased by 15-25% per year. The current demand for medicinal plant products, according to WHO,is approximately 14 Billion US dollars, going to be rising by 5 Trillion US dollars by the year 2050. The herbal medicines present in the "Nutraceutical" sector and now added food supplements approved by the FDA, estimated up to 5.1 Billion US dollars. In the US, the usage of medicinal plants increased from 2.5% - 30% in the span of 10 yrs. (from 1990 to 2000 years).

Out of 17 biodiversity countries in the world, India is one of them. Out of the 45000 species present globally, in India, people were using 4635 plant communities from 7500 species. Because of the diversity in climatic conditions, floral diversity is more in plants. In India only, the high usage of medicinal plants in different therapeutic systems can be seen.

India exports a large number of raw MAPs and processed plant products. India earned foreign exchange 53219 million US dollars by exporting plant drugs in the year 1994 – 95, and US-made 13250 million US dollars from essential oils **(**Lambert *et al.*, 1997). By exporting herbal materials and herbal medicines, India earned Rs.3000 Cr and crossed Rs.15000 Cr by 2015 (as per reports).

1.8 Plants under study:

Here we have taken three different medicinal plants to cure inflammations in humans. The three plants are *Phyllanthus emblica* (Indian gooseberry), *Terminalia chebula* (Myrobalan), and *Terminalia bellirica* (Bibhitaki). We are combining these three plants called 'Triphala', as we are combining the dry fruit extracts.

Scientific classification of *Emblica officinalis or Phyllanthus emblica* (Indian gooseberry):

Kingdom	Plantae
Class	Dicotyledons
Subclass	Monochlamydeae

Series	Unisexuales
Order	Euphorbiales
Family	Euphorbiaceae
Genus	*Phyllanthus*
Species	e*mblica*L.

Scientific classification of *Terminalia chebula*:

Kingdom	Plantae
Class	Dicotyledons
Sub Class	Polypetalae
Series	Calyciflorae
Order	Myrtales
Family	Combretaceae
Genus	*Terminalia* L.
Species	*chebula* Retz.

Scientific classification of *Terminalia bellirica*:

Kingdom	Plantae
Class	Dicotyledons
Sub Class	Polypetalae
Series	Calyciflorae
Order	Myrtales
Family	Combretaceae
Genus	*Terminalia* L.
Species	*bellirica* (Gaertn.) Roxb

1.8.1 Distribution and medicinal uses of "Triphala":

The combination of *Phyllanthus emblica, Terminalia chebula and Terminalia bellirica*is called "Triphala". All these plants are cosmopolitan in distribution. The "Triphala" is having significant usage right from the olden days. The best usage is present in Ayurveda. These three plants have their medicinal value. *Phyllanthus emblica* has an average height (1 to 8m) tree. The stem has branches 10-20cm long.

The tree is deciduous. The root system is a taproot. Leaves are simple, subsessile, tightly arranged on the branches, resembling pinnate leaves. The colour is light green, dorsiventral with a smooth margin and yellow or green-yellow flowers. The flowers are small, greenish-yellow in colour. The fruit is a berry, circular, light green, smooth surface, hard on appearance, with six vertical furrows. The raw or ripened, or dry fruit has medicinal values. The fruit is bitter in taste and astringent; later gives a sweettaste. The seed coat is hard, shell-like, six ribbed, split into three segments.

Fig.2 Amala or Amalaki *(Phyllanthus emblica L.*)

***Terminalia chebula*:** This is a wild tree, usually occurs in the Sub Himalayan regions to West Bengal and Assam. In the Himalayas, the plant can reach its maximum height of 1500m. *Terminalia chebula* is a wild-growing in the forests of Northern India, Central provinces and Bengal. It is common in Chennai, Mysore and Mumbai. Well-developed tap root long and highly branched stem with a simple smooth margin. The fruit ripens from November to March and falls soon after ripening. The fruit is a drupe, ovate with longitudinal wrinkles on it. After drying, the fruit becomes hard, stony, with a length of 2 to 4cm, breadth of 1.5 to 2.5cm with 5-6 longitudinal ribs. The fruit is harsh and astringent in taste with no odour.

Fig. 3 Harad/Haritaki (*Terminalia chebula*)

Terminalia bellirica: Wild plant usually grows in the forest. It is a deciduous tree, reaches 35 m height, sympodially branched. After the secondary growth, 10-20 mm thick bark formed. Leaves are simple, opposite or alternate, clustered at the tip of the branches, exstipulate, grooved at the tip, ovate with a pubescent surface on both sides when young, at maturity glabrous. Flowers are greenish-yellow in colour. The fruit is a drupe, ovoid with five ridges on it; after drying, it turns to hard and yellowish-brown colour fruit.

Fig.4 Baheda/Bibhitaki *(Terminalia bellirica*)

Phyllanthus emblica L: Number of uses of these medicinal plants mentioned in Indian System of Medicine. It acts to increase resistance as an antioxidant, controlling ulcers, anti-inflammatory, removing gastric problems, improves memory, lowers cholesterol level and decreasing ophthalmic disorder. When it combines with *Terminalia chebula* and *Terminalia bellirica*, it leads to the Arachidonic Acid pathway.

***Terminalia chebula*:** The uses were mentioned in the book "Indian System of Medicine". It is having a high ability for diuretic, homeostatic, cardiotonic, antitussive and laxative. They are used for cough, kidney and liver dysfunctions. When it is combined with *Emblica officinalis* and *Terminalia bellirica* to make Triphala, the medicinal uses enhance.

***Terminalia bellirica*:** The medicinal uses are mentioned in Ayurveda. It can protect the liver, treat respiratory conditions, controls cough, sore throat, controls eye infections. When combined with *Phyllanthus emblicaand Terminalia chebula*, it lowers cholesterol and prevents the death of heart tissue. "Triphala" is made by combining these three fruit extracts. Triphala powder is altogether used as an anti-inflammatory and anti-cancer. Triphala powder enhances the Arachidonic Acid pathway to suppress cancer causing genes.

1.9 Aim:

The present work aims to show the chemical action of the medicinal plants used for anti-inflammation and anti-cancer activity. The plants used in this listing in the Indian System of Medicine, i.e. “Ayurveda”. The work includes technique and interdisciplinary tools as phytochemicals, pharmacological chemical/bioinformatics, chromatography and many. Through this research, we are trying to give a similar value to the ancient medicinal herbs to the next generation. Different other technologies are used to achieve the aim and principle of the research work.

1.10 Objectives of the research:

- Identification of PLA_2, its inhibitory activity and metabolites from *Triphala.* Plant-based phytochemical and *in silico* study.
- On selected cells, the Anti-inflammatory and anti-cancer activity and selected metabolites with PLA_2 inhibitory activity.
- We are studying the Apoptotic and Cytotoxic effects on cancer cells by using anti-cancer compounds.

1.11 Hypothesis for research:

From the ancient period, people used to take plant extracts for different health problems. Ayurveda is the oldest and well-known medicinal system for Indians from the ancient period. As mentioned in the literature, the three traditional plants taken are *Phyllanthus emblica*, *Terminalia chebula* and *Terminalia bellirica* for treating and curing different health problems. The fruit powder of fruits of these three plants ismixed to make "Triphala". The powder was further analysed for different phytoconstituents, which show antimicrobial and other medicinal properties.

PLA_2 during chronic inflammation needs to be inhibited to avoid detrimental outcomes. Hence, formulating the potential inhibition, due to multitasking properties, efficacy for homeostasis, prevention and treatment of disease. The effect of Triphala is studied for inflammation and anti-cancer. A serious attempt has been made to elucidate the effects of Triphala and characterise its extracts to identify the potential bioactive natural product for inhibiting the PLA_2 activity, thus inhibiting the inflammation in several parts.

1.12 Research Gap in current knowledge:

All the plants under study are well documented for several uses in *Ayurveda* and belong to the *Ramayana* timeline. The prior art search on the subject shows that some phytochemical studies are reported. Several plants are considered for their inhibitory activity *in vitro* conditions. In theseconditions, the phytochemicals were extracted and preserved. Moreover, some of them are tested before using for treatment. We can use these plant extracts as bio-remediation. They are found to be targeting the Arachidonic acid (AA) pathway. These are developed for treating inflammations and cancer-causing cells. This process is under various stages, which include clinical development.

1.13 Statement of the problem:

Phospholipase A_2 inhibitory Activity of Triphala Plants Constituents for their Applications in the Treatment of Inflammatory and Oncology diseases.

CHAPTER - 2

LITERATURE REVIEW

2.1 Review on Triphala

From ancient times, in the Ayurvedic medicinal system, Triphala was used in treating gastrointestinal problems. The three-plant extracts of Triphala in different compositions are used for different problems. As Triphala is made from amla, myrobalan and bibhitaki, (*Phyllanthus emblica, Terminalia chebula* and *Terminalia bellirica*) it is having all the plant constituents and their medicinal values. It has Gallic acid, chebulinic acid, terflavin A, terchebin, ascorbic acid, phyllemblin and 2,4-chebulyl-β-D-glucopyranose. Triphala performs functions as an antioxidant plus agent for immunomodulation (Baliga M S *et al.,* 2012, Belapurkar P *et al.,* 2014). Triphala has many flavonoids (Luteolin), fatty acids, anthraquinones, saponins, amino acids and many carbohydrates. The microbiota present in the human gut transforms chebulic acid (a polyphenol present in Triphala) into bioactive metabolites to prevent oxidative damage (Lu K *et al.,* 2012, Olennikov D N *et al.,* 2015)

Triphala is an appetite stimulator, free radical scavenging, anti-inflammatory, antioxidant, prevention of dental problems, analgesic, antipyretic, gastric reduction, hyperacidity reduction, chemopreventive effect, chemoprotective, hepatoprotective, radioprotective, antistress, anti-carcinogenic, antimutagenic, wound healing, hypoglycemic, immunomodulating, antibacterial and anticancer effects. Triphala promotes food absorption, proper digestion, reduces bile duct relaxation, improves circulation, serum level, and cholesterol level, prevents immunosenescence, maintains homeostasis for the endocrinal system, enhance red cell count and hemocue.

2.1.1 Triphala action on digestive tract:

Triphala acts well on digestive tract health in humans and controls loose bowels in animals. By the increase in antioxidation values, Triphala secures the intestine (Biradar Y S *et al.,* 2007).Triphala protects the intestinal system of rats from blisters developed on account of stress **(**Nariya M *et al.,*2009). Apart from controlling digestive tract abnormalities, Triphala controls acidity, loose bowel problem, stomach

pain, mucid and betterment in stool evenness and occurrence. (Nariya M B *et al.,* 2011) Triphala controls inflammatory bowel disease in mice. All these effects are caused by Triphalas only by having flavonoids and antioxidant effects**. (**Rayudu V *et al.,* 2014)

2.1.2 Triphala regulates the stress levels:

Tension linked problems like excitement lead to cause adult disability. (Whiteford HA *et al.,*2010) Stress is such a severe problem that it disturbs other activities, may lead to chronic diseases. In animals, *Triphala* protects against induced cold stress and alterations in reversed induced stress behaviour. (Dhana Lakshmi S *et al.,* 2007) Triphala controls tension created by sounds. Triphaladirects the changes that occurs due to tension in rats by increasing immunity of the cells and antioxidant nature. The high-stress level is acquired by people due to the modern lifestyle. An extensive care is to be taken while using Triphala (Srikumar R *et al.,* 2006, Kumari N *et al.,* 2009).

2.1.3 The anti-obesogenic and anti-diabetic potential of Triphala:

In today's contemporary world, irregular food habits lead to obesity. Triphala can reduce stoutness, heaviness and crapulence caused by food. It controls the accumulation of bad fat, sugar levels in the body with a limited dose (Gurjar S *et al.,* 2012). Triphala inhibits pancreatic enzymes (like amylase, lipase, trypsin and proteases)breaks large polysaccharide molecules into glucose molecules. Triphala controls the digestion of starch and its absorption to decrease hyperglycemia. In the blood, polyphenolsreduce glucose and insulin. (Kamali S H *et al.,* 2012, Yang M H *et al.,* 2013)

2.1.4 Triphala for Cardiovascular health:

Hypercholesterolemia is the risk factor for cardiovascular problems. Triphala reduces low-density lipoproteins, levels of fatty acids and cholesterol levels. *Terminalia chebula*, in Triphala, controls the hypolipidemic effect in rats. These rats were fed on an atherogenic diet. Triphala effectively corrects the imbalance in the cardiovascular system. (Saravanan S *et al.,* 2007)

2.1.5 Antimicrobial potential of Triphala:

Water decoction of Triphala with ethanol shows its anti-microbial and anti-bacterial activity in humans suffering from the immunodeficiency virus. Triphala acts as a bactericidal, more effective on *Staphylococcus* species, less effective on *Pseudomonas aeruginosa* and *Salmonella typhi*. Triphala also shows antifungal actions for *Aspergillus species*. (Srikumar R *et al.,* 2007, Tambekar D H *et al.,* 2011)

2.1.6 The action of Triphala in Oral Care:

In the traditional Ayurvedic medicinal system, Triphalais used as an antimicrobial agent. Triphala efficiently reduces dental ache, oral bacteria and gingivitis in humans. (Prakash S *et al.,* 2014) Triphala is strong to eliminate *Enterococcus faecalis*. Mouth wash with Triphala can control cancer tendency caused due to tobacco. (Baratakke S U *et al.,* 2017)

2.1.6.1 Radioprotective effects of Triphala:

Triphala controls damage of reverse DNA and mutagenesis. Stopping damaging DNA is the first step to carcinogenesis. Triphala efficiently controls and reverse DNA damage and mutagenesis. Triphala effectively controls the induced (chemical or radiations) mutations. (Sandhya T *et al.,* 2006) Triphala efficiently controls the cut in the strand of plasmid DNA induced by gamma radiations. The gallic acid (a component in Triphala) is rich in phenolic compounds, which suppress free radicals in the body. (Naik G H *et al.,* 2005, Takauji Y *et al.,* 2016, Baliga M S *et al.,* 2013)

2.1.7 Anti-neoplastic activity of Triphala:

Triphala shows antineoplastic effects for various cancers as prostate cancer, bowel cancer, mammary gland carcinoma and cancer for the pancreas. (Baliga M S *et al.,* 2010) Triphala enhances cytotoxicity in cancer cells. It increases oxygen reactivity in intracellular spaces than in healthy cells. When mice treated with Triphala reduces the growth of tumour cells affecting ERK and p53 pathways, treating healthy cells with Triphala increases killer cells. So, Triphala acts as an antineoplastic agent, inhibitingthe capacity of the large intestine and several types of carcinomas. (Sandhya T *et al.,* 2006, Phetkate P *et al.,* 2012)

2.1.8 Triphala and its antioxidant nature:

The antioxidant nature of Triphala can maintain the health of all organs including the eye. In Triphala, amla is having more C-vitamin. Triphala extract is rich in antibiotics. Triphala reduces cataracts by the restoration of glutathione levels in the lenses of an eye. (Gupta S K *et al.,* 2010)

2.1.9 The action of Triphala on inflammation:

Triphala efficiently controls inflammation and is known as an anti-inflammatory agent. Triphala can remove the inflammatory marks and control cartilage and bone degradation. In the tissues of Arthritis, Triphala decreases lipid peroxidation and increases antioxidation levels.(Rasool M *et al.,* 2007) Triphala controls the generation of chemicals which cause swelling (COX) intracellular free radicals, inflammatory mediators (PGE2) and lysosomal enzymesare released efficiently. COX and 5-LOX cause inflammation and carcinogenesis which are controlled by chebulic acid (one of the constituents of Triphala). (Kalaiselvan S *et al.,* 2015)

2.1.10 Triphala controls ageing:

Triphala is used for decreasing the age of skin cells (antiaging). Human skin cell gene expression can be affected by Triphala. It forms the cellular antioxidant, SOD-2 genes by enhancing elastin (synthesizing gene), collagen-1and antioxidant genes. The phytochemicals present in Triphala controls the production of melanin and hyperpigmentation. Triphala takes out the free radicals from hydrogen peroxide, which causes senescence and damages cells. (Varma S R *et al.,* 2016)

2.1.11 Triphala used for different herbal therapies:

With a change in lifestyle and human health, the use of herbal therapies too changed. With a change in human lifestyle, digestion, absorption changed as well. Herbal usage can give remedies to these problems. The microbiota presents in the gut acts on the food components and enhances their bioactivity and bio absorption. So, these herbal products maintain human health and decrease chronic diseases. Herbal product Triphala improves absorption capacity and decreases the disease-causing tendency.

2.1.12 The action of Triphala on Gut microbiome:

The gallic acid and quercetin (phytochemicals) present in Triphala enhances the growth of *Lactobacillus* and Bifidobacteria. These microorganisms control the growth of *E.coli* (present in the gut). Gallic acid present in Triphala can be degraded by the enzymes present in Lactic acid bacteria (Eg.Tannase). Chebulic acid present in Triphalais converted into a metabolite like urolithin by the gut microbiota of humans. The urolithin can control oxidative damage.(Yadav S *et al.,* 2011, Tabasco R *et al.,* 2011, Jimenez N *et al.,* 2014)

Triphala shows good results in any age group to increase specific probiotic species. These probiotics increase digestion, bioabsorption and increase the bioactivity of Triphala.

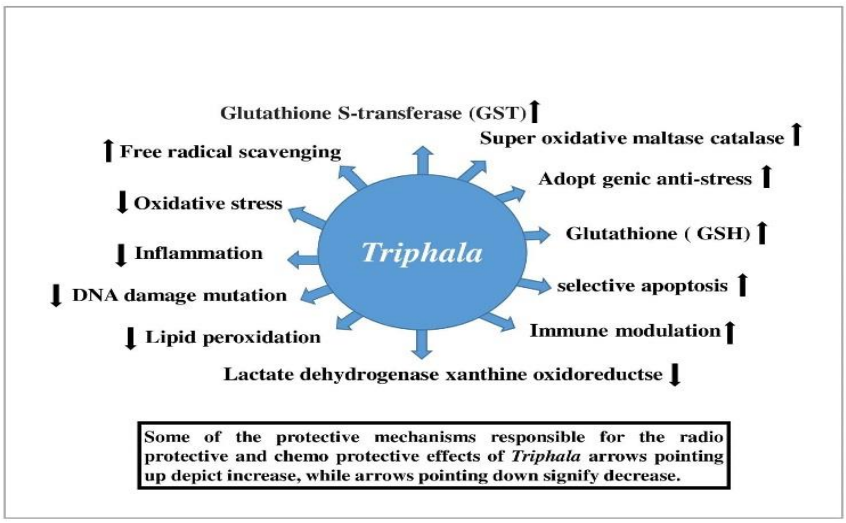


Figure.5 Triphala Protective mechanism

2.2 Knowledge of inflammation from Modern and ancient point of view:

In Ayurveda, the Traditional Medicines are extensively studied for different types of health problems and their recovery. The books written between 1500 BC and 600 AD (N. V. Krishna Kutty *et al.*) explained various health issues and inflammation in humans. These books are Charaka Samhita, Susruta Samhita, BrihatTrayee and AstangaSamgraha. Motivated by these books, in 700 AD (N. V. Krishna Kutty *et al.,*) Madhava Nidana wrote a book where a complete description of disease symptoms and inflammation was there, as a synopsis of the above books.

Inflammation has different names in Ayurveda as Utsedha, Shobha, Shotha; inflammation is considered as a symptom of disease, which gives pain and excess fluid accumulation is observed. (Kasinatha Sastry *et al.,* 1984)

According to the Modern Medicinal system, inflammation is considered as a healing process in an acute stage. When the cells are infected and this infection spreads to other cells, they too will be in a similar state. When the healthy tissues did not get a healthy state, then the infection may spread to other tissues too.

When this inflammation will be there for an extended period (Chung H Y *et al.,* 2009) damaging to the surrounding cells it is called chronic inflammation, Osteoporosis, Diabetes, Parkinson's disease and Arthritis are the best examples of chronic inflammations. In chronic inflammation, the levels of serum increase with an increase in the cytokinesis of the cells. Chronic inflammations may lead to cancer. In the 19^{th} century, Rudolf Virchow, a German physician, explained chronic inflammation and cancer with chronic cardio-vascular supply and diabetes. After that, the relationship between inflammation and cancer is known (Aggarwal B B *et al.,* 2011, & Chung H Y *et al.,* 2009)

In Ayurveda, the inflammation leads to less activity of the cells, more activity of tissues, new growth in the tissues, and the growth may be irregular (Kasinatha Sastri *et al.,*1984). Due to this uneven growth in inflammations, the supply of oxygen, nutrients and wastages will effect. Now, this may turn to form a tumour (benign or malignant). A toxic waste formation occurs with the blockage of micropores called Srotodushti. Due to reverse osmosis, differences between electrolytes occur. This type of waste formation will disturb the cell metabolic activities, leading to cancer. According to Ayurveda, another form of inflammation is a tumour. Based on the liquid present in the inflammation, it is considered as a standard or chronic type. If this effect the activity of other muscles and their lifestyle is changed is a sign of an oncological problem. (Sukta Das *et al.,* 2012)

2.2.1 The modern approach to inflammation:

Inflammation, according to Modern Medicine, is of 2 types acute and chronic; modern medicine says that inflammation is the healing process of the infected body. The interaction of the cells makes feasibility for repairing tissues, which occurs by uncontrolled cell division. If the inflammation is not controlled, then it may damage the host tissues too. (Kumar *et al.,* 2004)

Table.2 Comparison between acute and chronic inflammation

	Acute	Chronic
Causative agent	Damaged tissues	Continuous occurrence of inflammations where the infections cannot be controlled, disease-causing organisms keep growing.
Major cells involved	Neutrophils	Cells of mononuclear origin (monocytes, Macrophages, plasma cells, lymphocytes), fibroblasts
Primary mediators	Vasoactiveamines, eicosanoids	Development of IFN-y and other cytokines Factors, species of reactive oxygen, hydrolytic Enzymes
Onset	Immediate	occurrence is late
Time period	Few days	Infection occurs for a long time, sometimes years together.
Outcomes	Cure, the creation of abscesses, chronic inflammation	Destruction of tissues, fibrosis

2.2.2 Comparison between acute and chronic inflammation, (source Wikipedia)

2.2.3 Ayurvedic approach to inflammation:

Ayurveda says that the symptoms of inflammation are treated with suitable anti-inflammatory medicines. In the medicinal books, they described the fluid thoroughly and the inflammation caused by it. Inflammations are of two types, intrinsic and exogenous. We can observe an increase in the temperature and increase of the

pressure in the veins due to inflammation. (Kasinatha Sastri *et al.*,1984 Sutras Thana18/3, 18/4)

For inflammation, the treatments that are being followed presently were already given 3000 years back in Ayurveda. In Sushruta Samhita, they considered blood disorders as the cause of inflammation. They included another point, wounds cause inflammation. They were thus accepted by the Modern Medicine surgeons too.

In Charaka Samhita and Sushruta, Samhita discussed the swelling that occurred in the internal parts by inflammation because of the variations in the blood sugar. Obesity will increase this tendency. So this shows the relation between inflammation and obesity. Inflammation is directly linked to the metabolic disorders in the body.

According to the Medicinal books, the inflammation occurs internally (insensitive and essential organs) and externally (in muscle, skin and ligament joints). Inflammation is a symptom of an abscess. The abscess with inflammation occurs in different internal organs like the liver, heart, pelvic, spleen, bladder and kidneys in Ayurveda; inflammation enhances with more salt, irregular diet and lifestyle habits. Liver enlargement, diarrhoea, cough, abdominal diseases and anaemia cause inflammation. For any inflammation, infection or Krimi is the main reason.Ayurveda considers Tridoshaas the best one to explain the basic theory of disease.

The literature survey reveals that plant species of about 96 genera belonging to 56 families act against inflammations. Some of them are used in traditional systems of medicine with pharmacologically/therapeutically proven anti-inflammatory and antirheumatic claims are mentioned in table 3.

Table. 3 anti-inflammatory plants

Plant species	Trade names in India	Family
Aconitum napellus	Aconite	Ranunculaceae
Alpinia officinarum	Rasna	Zingiberaceae
Azadirachta indica	Neem	Meliaceae
Balanites roxburghii	Gari	Simaraubaceae
Boerhaaviadiffusa	Punarnava	Nyctaginaceae
Boswellia serrata	Salaiguggal	Burseraceae
Colchicum autumnale	Colchicum	Liliaceae
Commiphoramukul	Guggulu	Burseraceae
Curcuma longa	Turmeric	Zingiberaceae
Delonixelata	Vatanarayana	Leguminosae
Glycyrrhiza glabra	Liquorice	Leguminosae
Hedychium spicatum	Karpur kacheri	Zingiberaceae
Heliotropiumcurassavicum	Haatisura	Boraginaceae
Hemidesmus indicus	Margabi	Asclepiadaceae
Hibiscus rosa sinensis	Jassoon	Malvaceae
Indigofera aspalathodes	Hakna	Fabaceae
Inularecemosa	Poshkar	Compositae
Iris kashmiriana	Padma-pushkara	Iridaceae
Lawsoniainermis	Hena	Lythraceae
Leucas aspera	Hulkusha	Labiatae
Mammea longifolia (Vitexin)	Nagkesar	Guttiferae
Moringa oleifera	Sahinjan	Moringaceae
Morus alba	Tutri	Moraceae
Myrtus communis	Baragasha	Myrtaceae
Nepeta hindostana (Nepitrin)	Billilotan	Labiatae

Nerium indicum (Plumieride)	kaner	Apocynaceae
Nigella sativa	Kalonji	Ranunculaceae
Nyctanthesarbortristis	Seoli	Oleaceae
Nymphaea stellata	Nilkamal	Nymphaeaceae
Operculinaturpethum	Nakpatra	Convolvulaceae
Ougeniaoojeinensis	Sandan	Fabaceae
Paederiafoetida	Gandhali	Rubiaceae
Peltophorum	Kondachinta	Caesalpiniaceae
Phyla nodiflora	Jalapeepala	Verbenaceae
Piper longum	Pippali	Piperaceae
Pluchea lanceolata	Rasna	Compositae
Premnaherbacea	Bhumjambu	Verbenaceae
Prosopis spicigera	Shami	Leguminosae
Psoralea coryligolia (bavachinine)	Bakuchi	Leguminosae
Pterocarpus santalinus	Raktachandana	Leguminosae
Randiadumetorum	Mainphal	Rubiaceae
Salvadorapersicia	Brihatpilu	Salvadoraceae
Teramnus labialis	Mashaparni	Leguminosae
Tinosporamalbarica	Sudarsana	Menispermaceae
Urena lobata	Vanabhenda	Malvaceae
Vandal roxburgii	Rasna	Orchidaceae
Verbena officinalis	Karaita	Verbenaceae
Vitex negundo	Nirgundis	Verbenaceae
Withaniasomnifera	Ashwagandha	Solanaceae

Source: Some plants with anti-inflammatory activity (*The Useful plants of India,* 1988; *The Medicinal Plants of India,* 1987; The Wealth of India, 1952).

2.3 Knowledge on Cancer from Modern and Ancient point of view:

The description and treatment of cancer was present in Ancient Egypt therapies. The abscess that cannot be treated with medicines is called cancer. In 1600 BC, Edwin Smith Papyrus, in his book, described in detail cancer and removal of breast tumour by Cauterisation. He also specified that this disease does not have any treatment (American Cancer Society. 2009).

Hippocrates (460 BC – 370 BC) gave the word Carcinos (Greek word -crayfish) for different types of cancers. The term 'Carcinos' is also called Carcinoma. The veins in the tumour spread like feet of the crab, so the name is given as Carcinoma. The treatment is based on the fluid'stumour present in the body as yellow and black bile juice, phlegm and blood. Hippocrates against the traditions, described in Greek with drawings of the tumors on the nose, skin and breast. The treatment will base on tumour. The Greek physician Galen (2^{nd} century AD) gave 'oncos' for tumours, keeping 'carcinos' given by Hippocrates for malignant tumours. From Galen's word only, the name 'Oncology' is derived.(Moss Ralph W. *et al.,* 2004), (Karpozilos A *et al.*, 2004)

World Health Organization agency for carcinogens, The International Agency for Research on Cancer (IARC), gave the total number of people suffering, cured and died in the world. It identified that over 40 million people (above 15yrs) identified as suffering from carcinoma in the last 5-6years. Repeatedly occurring carcinomas are breast, lung and colorectal cancers. Maximum people lost their lives due to liver, stomach and lung carcinoma. People getting affected with cancer and their death rate may have a drastic increase by 2025 with the increase in the global population.

Cancer occurs due to irregular division and cell growth. It may lead to invading nearby tissues resulting in malignant tumors. It will spread to other tissues or parts through blood or lymphocytes. Inflammations or tumors never turn into cancers; they are benign. Benign tumors never infect other glands or tissues. Characteristics features of malignant tumors are

(1) Mass of cells signalling
(2) Controls growth suppressors
(3) Controls death of the cell
(4) Gives a good life for the cells to replicate
(5) Producing new blood cells
(6) Activating invasion and metastasis.

2.3.1 Cancer Classification

Cancers are classified based on the origin and grouped into six varieties. They are:
(1) Carcinoma (2) Sarcoma (3) Myeloma (4) Leukaemia (5) Lymphoma (6) Mixed Types

Carcinoma: Carcinoma types of cancers are developed by the unlimited division of outer cells. Breast cancer, lung, pancreas, prostate and colon cancers belong to carcinoma type. In overall cases of cancers, we can see carcinoma type is nearly 85%. Majorly this is divided into two kinds, adenocarcinoma and squamous type of carcinoma. Adenocarcinoma arises on the gland or organ and squamous type appears on the epithelial cells of squamous.

Sarcoma: Unlimited cell division occurs in other parts of the body but not in bone marrow is called sarcoma. These occur in bones, cartilage bones, nervous system, fat tissues etc.

Sarcoma: Cancer grows outside the bone marrow in free thesaurus cells. It may arise in different areolar tissues like bone, nerves, cartilage, fat etc.
Examples.
• Rhabdomyosarcoma (skeletal muscle)
• Mesothelial sarcoma
• Angiosarcoma or hemangioendothelioma (blood vessels)

Myeloma:

Cancer grows on the bone marrow plasma cells. These cells release some proteins in the blood, which leads to the spread of cancer to other parts.

Lymphoma:

Lymphoma or apathetic cancers arise in distinct organs of human beings like inthe brain, breast or stomach. These grow as a solid mass in these organs. Apathetic cancers are of two types, Hodgkin and Non-Hodgkin. Sternberg-Reed cells are characteristic of Hodgkin lymphoma, which distinguishes it from Non-Hodgkin lymphoma.

Leukaemia:

Leukaemia, referred to as liquid cancers, usually occur in the bone marrow. Leukaemia is derived from the Greek word, which means "White blood". Due to this disease, many immature white blood cells are produced. Thuspatients are exposed to diversified health issues. When the number of WBC increases, then red blood cells lose clotting capacity. When a cut occurs, lot of blood is lost resulting in ananaemic condition. Where Leukaemia develops or at which age it occurs in the body, it is divided into two types. They are Acute myeloid Leukemia and chronic myelocytic Leukaemia, the first one starts in Bone marrow and the second one at any age. Leukaemia infects people at different ages, and some infect only blood cells.

Mixed Types

Mixed type of cancers includes one or more types of Leukaemia. Examples for this are:

- Squamous cell carcinoma
- Mullerian Mixed Tumor
- Metastatic tumour
- Dysgerminoma

2.3.2 Causes of Cancer

With the change in the environment 90-95%, we may get cancer and genetically only 5-10% chance. The cancers caused by the environment are not inheritable. Through

the environment means, by the tobaccosmoke, change in diet, obesity, stress, radiations or by inflammations.

Research observations related to Cancer

1. The factors (internal or external factors) that manage to change genes or their functions may result in abnormal behaviour causing tumors.
2. With the change in the genes caused by diet.
3. Eventually, genetic variations and their consequences in the cell leads to deviation in the entire organism.

Diagnosis

The symptoms or signs can identify cancers, which can be recognised by a screening test. These patients should undergo medical tests like blood test, diagnosis of tissue, Endoscope, CT & MRI scan and X-ray.

Pathology

Once the tumor is diagnosed in the tissue, then it gives clarity as to how the cells multiplied, any abnormalities in the genes and other features too. Thus pathology helps out in giving correct treatment to the patient. The latest pathological tests provide a clear picture of molecular changes as a fusion of genes, mutations and numerical changes in the chromosomes. These problems need a different kind of treatment.

Treatment

Cancer treatment requires a multi-prong strategy but the approach to fight cancer is entirely based on its type; radiation therapy, Surgery and Chemotherapy which are the most practised and successful procedures to deal with this dreadful disease.

Surgery

The first Cancer treatment is surgery. During this, other tissues are cut along with the affected part.

Radiation

It is another method to treat cancer, involving ionising radiation. With this type of treatment, the cancer cells are permanently killed or some relief is given to the patients. The patients who are in the first stage of cancer can be handled. This treatment is not preferred for all kinds of cancers. The radiation method is given to the patients after the surgery or chemotherapy treatments. For neck cancer and head tumors, only radiation treatment is provided. Many patients feel that radiation is the best method in treating bone metastasis, where severe pain occurs. This treatment gives maximum relief to the patients.

Chemotherapeutic:

After the surgery, a chemotherapeutic treatment was used for some cancers for better results. Cancers like mammary cancer, bowel cancer, Carcinoma ofthe pancreas, malignant bone tumor, scrotum cancer, Carcinoma for ovaries and bronchogenic Carcinomas are treated with chemotherapy.

Based on the intensity of the disease, chemotherapy is given. With chemotherapy, the useful tissue is also damaged. Then,one realises the importance of natural products. Plants have remained a vital source of drugs in third world countries, as monetary constraints are leading them to move towards homemade remedies. India is categorised as a developing nation; more than 20% of the population remains, depend on herbs. Most of the researches on terrestrial plants /organisms are already done. It is still on, have yielded impressive lead for treating different diseases, either compound isolated are directly used as drugs, in a limited number of cases their mimics have been developed by chemists for use.

2.4 Natural Products and Cancer

2.4.1 Plant extracts used as agents for controlling cancer

Extracts from the plants or the plant parts are divided into two categories depending upon the source:

1.Terrestrial plants /organisms

2. Marine plants/organisms

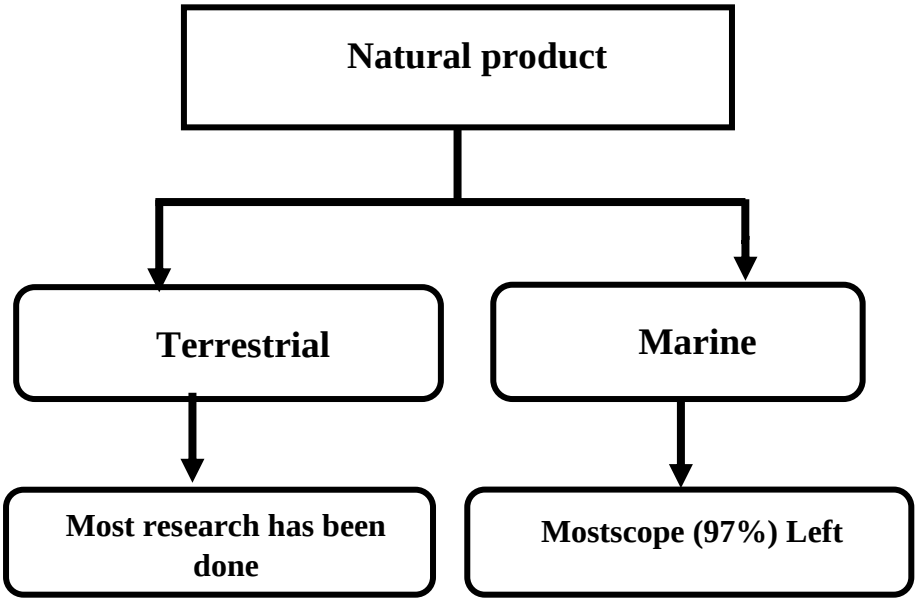


Fig. 6. Natural Product Classification

Table.4 List of some important anticancer plants

PLANT NAME	FAMILY	ACTIVE PART
Acacia xanthophloea	Leguminosae	Fruit
Adenium obesum	Apocynaceae	Leaf
Adiantum macrophyllum	Pteridaceae	Entire
Aeonium arboretum	Crassulaceae	Leaf
Aglaia foveolata	Meliaceae	Fruit
Alnus japonica	Betulaceae	Wood
Aphanamixispolystachya	Meliaceae	Stembark
Arisaema erubescens	Araceae	Root
Arisaema erubescens	Araceae	Root
Aster amellus	Asteraceae	Entire
Carapaguianensis	Meliaceae	Seed oil
Combretum caffrum	Combretaceae	Bark
Coriolus versicolor	Polyporaceae	Fruitbody
Crassocephalumbojeri	Asteraceae	Entire
Croton lechleri	Euphorbiaceae	Latex

Cyathea fauriei	Cyatheaceae	Shoot
Dillenia suffruticosa	Dilleniaceae	Fruit
Dioscoreacollettii	Dioscoreaceae	Rhizome
Dysosmapleiantha	Berberidaceae	Root
Echinopsgrijisii	Asteraceae	Root
Echinopslatifolius	Asteraceae	Root
Echitesvucatanensis	Apocynaceae	Latex
Euphorbia ebracteolata	Euphorbiaceae	Aerial parts
Euphorbia heterophylla	Euphorbiaceae	Stem
Euphorbia kansui	Euphorbiaceae	Root
Euphorbia marginata	Euphorbiaceae	Entire plant
Euphorbia micractina	Euphorbiaceae	Entire
Euphorbia prolifera	Euphorbiaceae	Latex
Ficus pretoiae	Moraceae	Sap
Fissistigmaoldhamii	Annonaceae	Stem
Hedyotischrysotricha	Rubiaceae	Entire
Hypericum japonicum	Guttiferae	Entire
Hypoxisrooperii	Hypoxiaceae	Tuber
Inulalinariaefolia	Asteraceae	Flowers
Ipomea batata	Convolvulaceae	Rhizome
Juglans mandshurica	Juglandaceae	Root
Juncus acutus	Juncaceae	Leaf
Lavandula angustifolia	Meliaceae	Leaves
Leptadenia hastate	Asclepiadaceae	Bark
Leptadenia hastate	Asclepiadaceae	Bark
Ligustrum lucidum	Oleaceae	Seed
Maackia tenuifolia	Leguminosae	Root
Maytenuscanariensis	Celastraceae	Fruit juice
Maytenus serrata	Celastraceae	Seed

Melaleuca alternifolia	Myrtaceae	Leaves
Melastomamalabathricum	Melastomataceae	Flower
Monninaobtusifolia	Polygalaceae	Aerial parts
Ocotea foetens	Lauraceae	Branchlets
Phymatosorusdiversifolium	Polypodiaceae	Root
Phytolacca esculenta	Phytolaccaceae	Root
Pinus parviflora	Pinaceae	Strobilus
Plantago asiatica	Plantaginaceae	Leaf
Pratianummularia	Campanulaceae	Entire
Rabdosiarubescens	Labiatae	Leaf
Ruellia tuberose	Acanthaceae	Bark
Salvia chinensis	Labiatae	Entire
Salvia officinalis	Labiatae	Leaves
Scirpusholoschoenus	Cyperaceae	Inflorescence
Scutellaria indica	Labiatae	Root
Sedum alboroseum	Crassulaceae	Entire
Swietenia humilis	Meliaceae	Seed
Tabebuia impetiginosa	Bignoniaceae	Stem bark and trunk wood
Tabebuia rosea	Bignoniaceae	Stem bark and trunk wood
Tabebuia serratifolia	Bignoniaceae	Stem bark and trunk wood
Thalictrum fabri	Ranunculaceae	Root
Thevetiaahouia	Apocynaceae	Leaf and Stem
Thevetiagaumeri	Apocynaceae	Leaf and Stem
Thevetiaperuciana	Apocynaceae	Leaf and Stem
Uncaria tomentosa	Rubiaceae	Bark
Viscum album	Loranthaceae	Leaves
Viscum calcaratum	Loranthaceae	Entire
Ziziphus mauritiana	Rhamnaceae	Stem bark and Fruit

Source: Medicinal Plants and Their Extracts Used in Treating Cancer-Causing Cells (Srivastava Shishir*et al.*, 2016)

2.4.2 Salt water organisms act as anti-cancer agents:

The maximum number of bioactive molecules extracted from salt water organisms and plants. The saltwater organisms yield more fluorescent secondary secretions than terrestrial one. The specific fluorescent once are alkaloids. Microorganisms occur in large number in marine habitats. This growth is beyond our estimation as water occupies 70% of the Universe, species variegation is seen in the marine condition. These microorganisms exhibit a lot of bio-diversity when compared to general microorganisms. These microorganisms are the primary producers of alkaloids in the Universe. These microorganism's production is in the deepest regions of the oceans, deep-sea vent groups and narrow ocean regions. All these areas cover less than one per cent of the earth's surface, but these organisms are rich in species and produce biological products of the world. From these microorganisms, many drugs are discovered. Among them, anti-cancer drugs are developed from different anti-cancer agents.

Table.5 List of marine Anti-cancer agents.

Compound	Organism	Class of Compound	Mechanism of Action
7-Dehydrocholesterol	Algae	Triterpene	Increases in cell death induction in estrogen receptor-negative breast cancer cells
Aaptamine	Sponge	Alkaloid	p21 is introduced and cell divisions were stopped
Aeroplysinin	Sponge	Alkaloid	Cell death starts in the multiplied endothelial cells.
Alkylpyridinium	Sponge	Alkaloid	Cell death was initiated and cell attachment was decreased
Aplidine	Ascidian	Depsipeptide	Induction of apoptosis with concomitant G1 arrest and G2 blockage
Aplidine	Ascidian	Depsipeptide	Oxidation and inactivation of tyrosine phosphatase activity
Asciddemin	Ascidian	Alkaloid	Direct iminoquinone reduction and reactive oxygen species generation
betadine 6	Sponge	Alkaloid	Inhibition of angiogenesis in vitro and in vivo involves apoptosis
Bryostatin-1	Bryozoan	Macrolide	Potentiation of ara-C induced apoptosis by PKC-dependent release of TNF-α

Cambridge 800	Sponge	Alkaloid	Induction of erythroid differentiation and cell cycle arrest
Chondropsin A	Sponge	Macrolide	In Vitro inhibition of V-ATPase enzyme
Clavulone II	Soft coral	Prostanoid	G1 cell cycle arrest and apoptosis
Cortistatin A	Sponge	Alkaloid	Selective inhibition of angiogenesis
Diazonamide-A	Ascidian	Peptide	Disruption of mitosis and cellular microtubules with inhibition of GTP hydrolysis
Dictyostatin	Sponge	Polyketide	Induction of tubulin polymerization
Dideoxypetrosynol A	Sponge	Fatty acid	Induction of apoptosis via a mitochondrial signalling pathway
Dolastatin 10	Mollusc	Peptide	Binds to the amino-terminal peptide of β- tubulin containing cysteine
Dolastatin 11	Mollusc	Peptide	F-actin stabilization by the connection between two long-pitch strands
Ecteinascidin-743	Ascidian	Isoquinoline alkaloid	Telomere dysfunction increases susceptibility to ET-743
Fucoxanthinol	Ascidian	Carotenoid	Induction of apoptosis
Geodiamolides	Sponge	Peptide	Disorganization of actin filaments
Girolline	Sponge	Alkaloid	Induction of G2/M cell cycle arrest and p53 proteasome recruitment
Hemiasterlin	Sponge	Tripeptide	Induction of microtubule depolymerisation
Ircinin-1	Sponge	Sesterterpene	G1 phase inhibition and apoptosis induction
Kahalalide F	Mollusc	Depsipeptide	Potent cytotoxicity and induction of necrosis
Lamellarin D	Mollusc	Alkaloid	Alkaloid ErbB3 protein and PI3K- Akt pathway involved in necrosis induction
Lexaphycins A and B	Bacterium	Cyclic peptides	Increased polyploidy by putative topoisomerase II alterations
Leptosins C and F	Fungus	Alkaloid	DNA topoisomerase I and II inhibition and apoptosis induction

Lissoclinolide	Ascidian	Fatty acid	G2/M cell cycle arrest
Onnamide A	Sponge	Polyketide	Protein synthesis inhibition
Philinopside A	Sea cucumber	Saponin	Inhibition of angiogenesis and receptor tyrosine kinases
Psammaplin A	Sponge	Alkaloid	Inhibition of aminopeptidase and suppression of angiogenesis
Variolin B	Sponge	Alkaloid	Inhibition of cyclin-dependent kinases and apoptosis induction

2.4.3 The microorganism is the significant source for preparing anti-cancer compounds:

Plenty of pharmaceutically important agents were isolated from microorganisms.The immunosuppressive potential of Rapamycin from *Streptomyces* hygroscopicus is a well-documented example. It hampers the next steps in cell division from the mid to late G1 stage in T & B cells.

Table.6 List of Microorganism as anti-cancer agents

Compound	Microorganism	Used in Cancer
Epirubicin	*Streptomyces pneuceticus*	Breast cancer
Idarubicin	*Streptomyces pneuceticus*	Breast cancer and leukaemia
Daunomycin	*Streptomyces coeruleorubidus*	Leukaemia
Doxorubicin	*Streptomyces pneuceticus*	Lymphoma, breast, ovary, lung and sarcomas
Actinomycin	*Streptomyces spp.*	Sarcoma and germ-cell tumors
Bleomycin	*Streptomyces verticillus*	Germ-cell, cervix and head and neck cancer
Rapamycin	*Streptomyces hygroscopicus*	Experimental
Geldanamycin	*Streptomyces hygroscopicus*	Experimental
Mitomycin C	*Streptomyces caespitosus*	Gastric, colorectal, anal and lung cancer

Source: Anti-Cancer Activity of Compounds Derived from Selected Indian Medicinal Plants (Srivastava Shishir *et al.*, 2016)

2.4.4 An insight to cancer occurrence:

With the changes in DNA sequences, sometimes the cell behaves abnormally (Flora *et al.,* 1996). The other factors (outside the cell) play a significant role in its division and its original activity (Luca, A.H *et al.,* 2003). It results in varying behaviour for the cell. These variations in the cell structure and function may occur in carcinoma.

2.4.5 List of the factors for showing variations in the structure and function of the cell:

Eternal:

The age of the primary cell is terminable. However, carcinoma cells release some enzymes, which make the original cells divide unrestricted (Cheng *et al.*, 2004).

Following the original signals for divisions:

All the cells obey the signals from the gene for dividing. Nevertheless, carcinoma cells do not obey the signals and divide unrestricted by producing different proteins. These proteins release signals for unlimited divisions (Liou *et al.,* 2011).

Not following the stop indicator:

Carcinoma cells ignore the pause indications for cell divisions, and it keeps on multiplying (Sluis *et al.,* 1994).

Controlling cell lysis:

Initially cells undergo lysis when they are unable to withstand the tension created inside. However, carcinoma cells can withstand the pressure by increasing the number of cells.

Formation of new blood vessels:

Carcinoma cells initiate the creation of new blood vessels to have a better molecular supply. These molecules are absorbed as the carcinoma cells vigorously multiply.

The presence of carcinoma cells at various places:

Finally, the carcinoma cells terminate this process by being present at different locations in the body from their starting point.

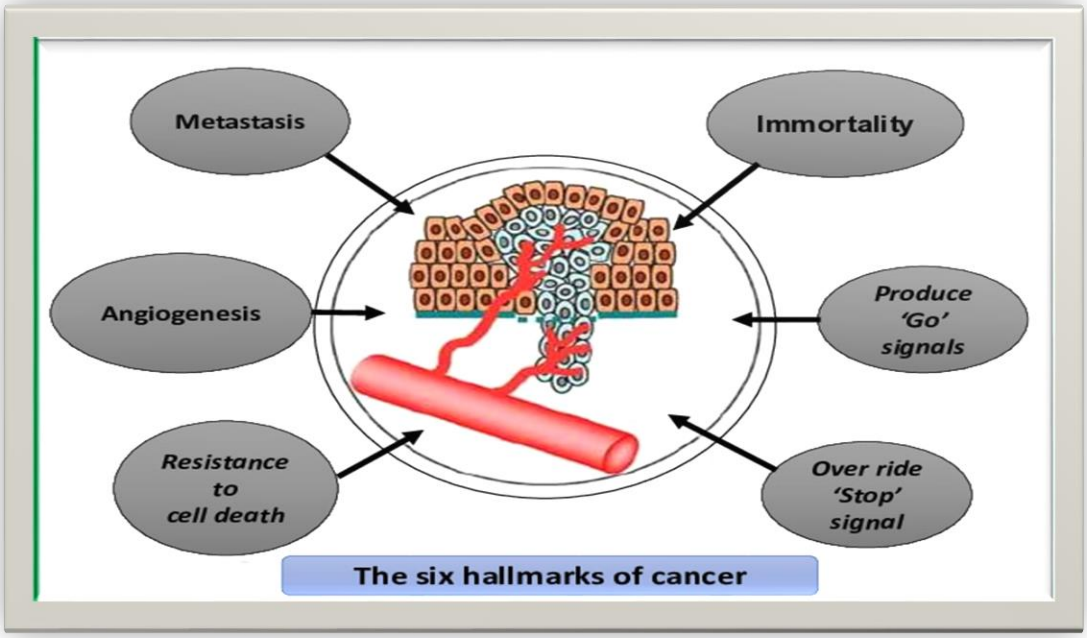
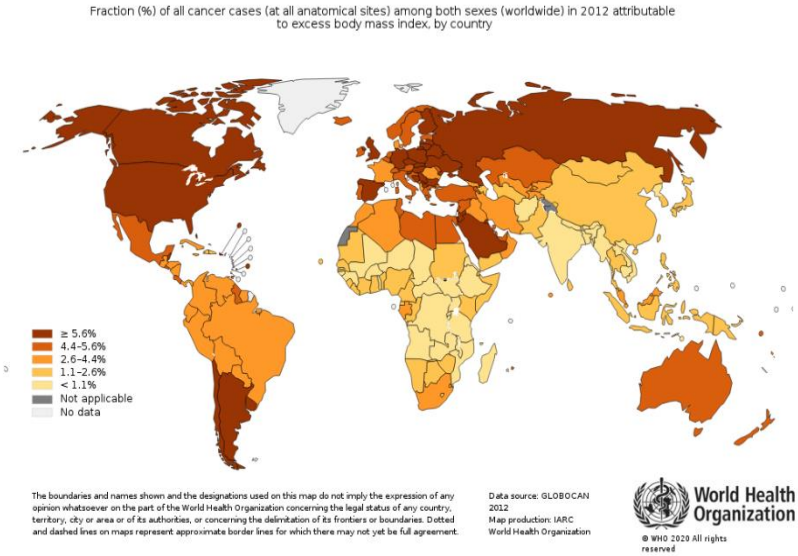


Fig. 7 Hall Marks of cancer

2.5 Breast Cancer

This is found mostly in women across the world. Though it can be cured by chemotherapy and radiations, it is better to use preventive measures like getting mammography checks done regularly. But this has a lot of undesirable side effects like loss of hair, appetite and mutation of functional cells; hence herbal products are accepted all over the world for their non-existent side effects as well as being readily available and economical. However, when breast cancer is concerned, though plants have shown promising anti-cancer effects when tested *in vivo* and *in vitro*, it has not been completely confirmed as clinical tests are not available. In the present senario the cause and impact of breast cancer are found mostly in industrialized and developing countries.(Rice S *et al.*,Whitehead SA *et al.*,2006) This is because of environmental and lifestyle factors and not related to genetic factors, as previously believed (Anand P *et al.*,2008). There are various factors to thislike early puberty, nulliparous, late pregnancy, late menopause (Mørch LS *et al.,* 2017).Besides this, the use of drugs-related to hormone replacement etc., are the main cause / risk factors resulting in breast cancer. In maximum cases, it leads to death. (Lambertini M *et al.*,

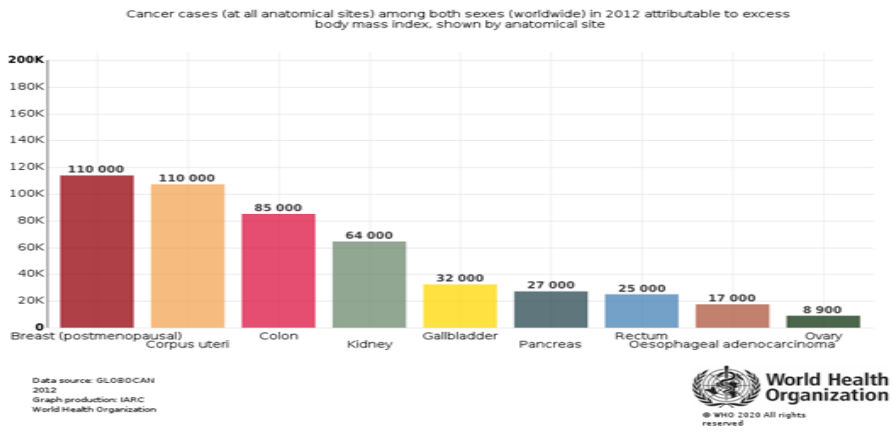
2016) There is a vertical rise in cancer-related causes which has alarmed IARC (international agency for research on cancer).



(Source: American cancer society**)**

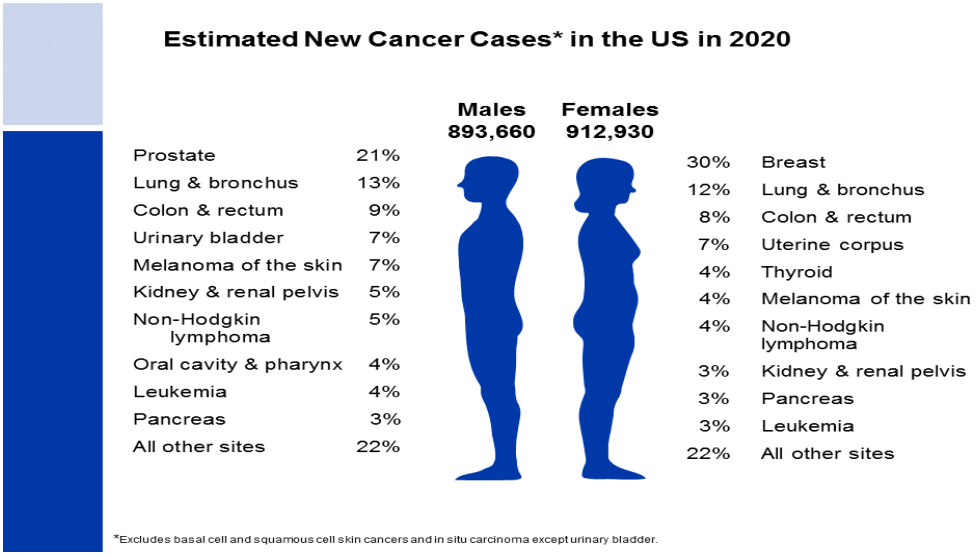
Figure 8 World Wide Percentage of Cancer

Across the globe 0.62 million females dies with breast cancer onlyout of 11.6% of cancer cases, as reported by IARC (Bray F *et al*., 2018). Based on the assiduous study affected people may reach 3.05 million by 2040, and the death toll may reach 6.99 million (Ferlay j *et al.*, 2018). Out of everyten women, one woman may get breast cancer at some phase of her life (Caffarel M M *et al.*, 2016).



(Source: American cancer society)

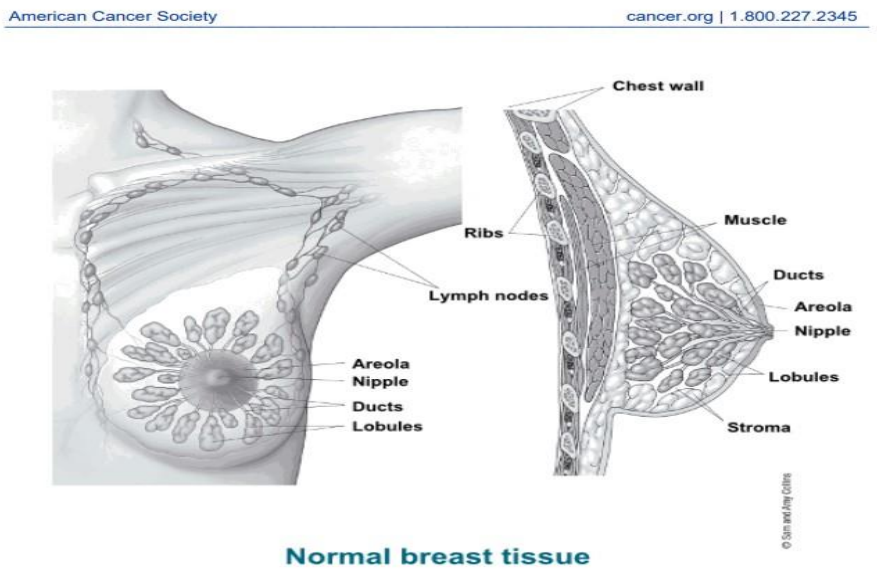
Fig.09 Percentage of different cancers world wide

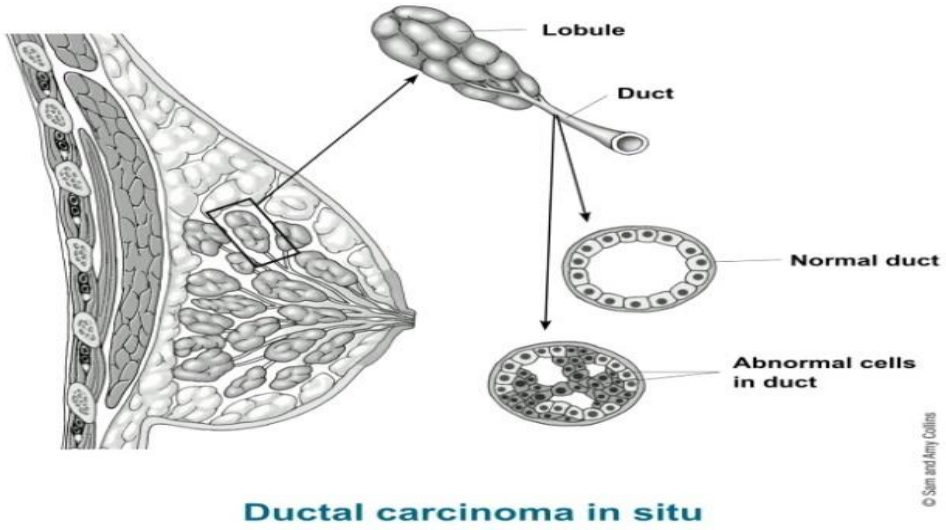


(Source: American Cancer Society journal, CA: A Cancer Journal for Clinicians. The Facts & Figures annual report)

Fig.10 Percentage of new cancer cases in USA 2020

In 2020, more than 1.8 million people are effected by cancer. In that, 21% are male affected with prostate cancer, 13% with lung cancer and 9% with colorectal cancers. In females, 30% with breast cancer, 12% with lung cancer and 8% with colorectal.





(Source: American cancer society)

Fig. 11 Shows Ductal Carcinoma in Situ

2.5.1 The onset and treatment of Breast Cancer:

Breast cancer alters the DNA strands or controls the protein synthesis. This increases the cell growth in that area (Hanahan D *et al*., 2011). With this, the change in the shape of the organ occurs, which finally can spread to other parts of the body (Lin W *et al.,* 2007).

The frequent treatment used is chemotherapy and radiation, which does help in the survival rate but with many drastic side results, and in some cases can be life-threatening.It is also very expensive as aftercare is mandatory in such cases.

2.5.2 Toxicity in the treatment of Breast Cancer

Cancer treatment by using chemotherapy is not very effective in certain patients. This is because of its side effects. These side effects are dangerous in some cases and might be normal in certain other cases (Middleton J *et al.,* 2018) the use of List of the drugs used in chemotherapy and their side effects:

Table.7 List of the drugs used in chemotherapy and their side effects:

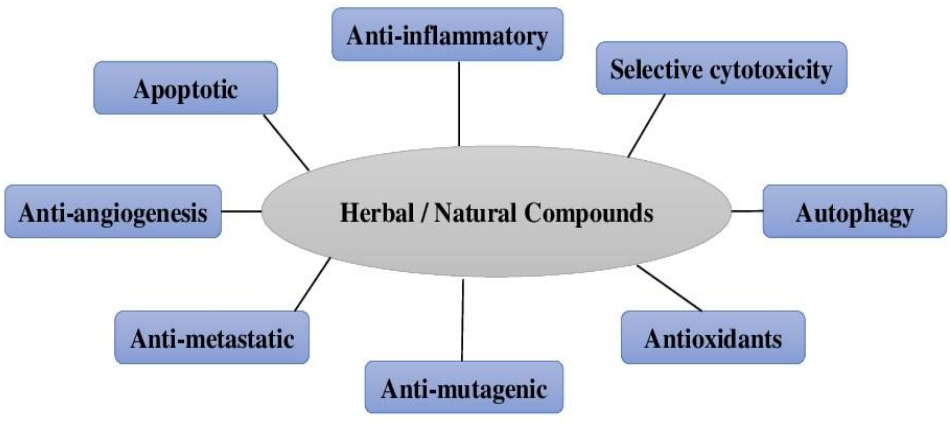
Drugs	Common side-effects
Doxorubicin	Cardiotoxicity, infertility, alopecia, nausea & vomiting, low blood counts.
Daunorubicin	Alopecia, nausea & vomiting, mouth sores and low blood count may cause infertility and cognitive heart failure on exceptional occasions.
Epirubicin	Increased risk of infectious diseases, hair loss, respiratory problems, and decreased blood count.
Cisplatin	Nausea & vomiting, kidney toxicity, ototoxicity and decreased blood count.
Paclitaxel	Alopecia, pain in joints and muscles (arthralgia and myalgia), peripheral neuropathy, nausea & vomiting, diarrhoea and hypersensitivity.
Cyclophosphamide	Temporary hair loss, nausea & vomiting, poor appetite, discolouration of skin and nails, low blood count, loss of fertility.
Tamoxifen	Cardiotoxicity, respiratory difficulties, abnormal vaginal bleeding, tenderness and numbness in the face, hands and legs.
Raloxifene	Hot flashes, flu, joint and muscle pain, rhinitis and blood clots, including deep vein thrombosis in rare cases.
Herceptin	Flu-like syndrome, respiratory problems, insomnia, hypersensitivity, cardiotoxicity, peripheral neuropathy, alopecia, low blood count, nausea & vomiting.
Gefitinib	Eye irritation, hypersensitivity, poor appetite, nausea & vomiting, pulmonary and respiratory problems, liver toxicity.
Bevacizumab	Upper respiratory infection, alopecia, nausea & vomiting, abdominal pain, constipation, nose bleeding, proteinuria and in rare cases, cognitive heart failure and nephrotic syndrome were observed.
Capecitabine	Low blood count, risk of infection, hand-foot syndrome, hepatotoxicity, eye irritation, nausea & vomiting, poor appetite and constipation.

Source: (Herbal Remedies for Breast Cancer Prevention and Treatment http://dx.doi.org/10.5772/intechopen.89669)

Anther reasonfor usage of low dosagein order to avoid annihilation of health cells and critical organs and thus have two therapeutic indexes (Weinberg SE *et al.*, 2015). There is a dire need for natural compounds for curtailing cancerous growth by disrupting healthy cells.

2.6 Herbal compounds:

Since the evolution, identification and awareness of diversified plants were based on their products. In developing countries, we can see the drastic hike in the demand for the medicinal plants used today when compared to the earlier days, i.e., traditionally. This is because of their low cost, readily available and without any side effects; some bio-chemicals can effectively control cancer. Example: Curcumin, Quercetin etc. These are highly harmful to the diseased cells and not harmful to the normal cells (Yuan H *et al.*, 2016). Naturally occurring compounds, they show a variety of anti-cancer activity, anti-oxidant, anti-inflammatory, anti-mutagen, and apoptosis-inducing activity, which helps prevent the growth and spread of cancer at the early stage.



Features of herbal compounds that attribute to their anticancer activity.

Source: (Herbal Remedies for Breast Cancer Prevention and Treatmenthttp://dx.doi.org/10.5772/intechopen.89669).
Fig. 12 Herbal compounds that attribute to their anticancer activity

Diet is similarly critical in the early stages and consumption of astonishing range of herbals in adequate quantities facilitates further growth bydisturbing cell cycle, sometimes leads to the cell death, controls the cancerous cell formation, restricting certain enzyme release which leads to tumor cell formation and blockading the signalling pathway that is important for most cancers' development (Huang W Y *et al*., 2010)

Between the years 1981 and 2014, out of the 136 anti-cancer drugs released, 83% were herbal compounds or their derivatives (Amaral R G *et al.,*2019). Among them, the commonly used once include vinblastine, vincristine, docetaxel and paclitaxel (Zyad A *et al.,* 2018).After controlling cancer tendency in the breast by the chemicals obtained from plants, no much progress has been made in clinical trials, and unless and until significant efforts are made, the treatment by the low toxic herbal products come to nought.

The phenolic compounds (Ex. Anthocyanin, Phenolic acids etc.) efficiently control and prevent cancer (Huang W Y *et al*., 2010). They're observed abundantly in life; those compounds effectively contribute toward carcinogenesis mechanism and chemo preventive sports (Ambriz-Perez D L *et al*., 2016).

2.7 Antioxidants:

After the much escalation of the role of free radicals & antioxidants in several diseases, it is relevant to know the purpose of the elimination process. Nowadays, disease means fluctuations in blood pressure and heartbeats. This is because of the imbalance between antioxidants and pro-oxidants. Pro-oxidants occur by the release of a greater number of free radicals. The free radicals are released more due to the stress or release of antioxidants with improper diet (Rakesh and Rajesh *et al.,* 2006). So, it is very important in maintaining the number of oxidants in the body.

Nowadays, herbal medicine usage is increased both in developed and undeveloped countries because of their production and less side effects. (Khopde *et al.,* 2001; Naik *et al.,* 2003). The practice of using plant extracts was the oldest tradition named as

Ayurvedic system. In this Ayurvedic system, they explained the usage of plant extracts for all different types of problems. The plant extracts can control mental and physical health and make the body healthy from different problems. Free radical means having one free electron in the outermost orbits. These free electrons easily pair up with any carbohydrate, lipids and proteins to wipe out the cell. The free-electron may shift between molecules to make the donor neutral, and the molecule which receives electrons has a free radical in it, some cells generate free electrons for defensive purpose. Live cells produce free radicals to remove toxic substances and neutrophils make free electrons to destroy the microbes (Lunec*et et al.*,2002). Maximum free radicals released in mitochondria damage the mitochondrial membrane and DNA of mitochondria (Rakesh and Rajesh *et al.,* 2006). When the free radicals are more than required, they destroy tissue, subject to the environment (ultraviolet radiations, pollutants, and smoke) may harm tissues or may mislead seekers of free radicals. It may lead to trauma, which is linked to many disorders like Rheumatoid Arthritis, diabetes, heart failure, inflammations, hypertension, cancer and many more (Chen *et al.,*2002). When free radicals exceed their minimum number in the human body, antioxidants neutralize them by detoxification. This process is called oppression (Kohen &Nyska *et al.,* 2002).

Formation of free radical:

Radical formation by electron transfer: $A + e^- \rightarrow A^{\cdot-}$

Radical formation by homolytic fission: $X{:}\ Y \rightarrow X^{\cdot} + Y^{\cdot}$

Ion formation by heterolytic fission: $X{:}\ Y \rightarrow X^- + Y^+$

2.7.1 Free Radical types:

The free radicals are mostly Reactive Oxygen Species (ROS) and Nitrogen free radical. These two have superoxide (O2-), hydroxyl (•OH), peroxyl (RO2•), alkoxyl (RO•), hydroperoxyl (HO2•), nitric oxide (NO) and nitrogen dioxide (•NO2) radicals. The two free radicals, i.e., Nitrogen and oxygen, quickly metamorphose to non-radical reactive species. Such as hypochlorous acid (HOCl), peroxynitrite (ONOO-)

and hydrogen peroxide (H2O2). With regular activity, the body produces more number of ROS and antioxidants delimits them by their protective action.

A change in the oxidation by intracellular or cellular molecules by the differences in ROS and antioxidants (Duh *et al.,* 1999). An antioxidant inactivates the ROS attack and stops the oxidation that occurs by free radicals. It is useful to prevent the various types of infections (Havsteen *et al.,* 2002; Hertog *et al.,* 1995). Superoxide free radical anion is produced by transferring a single electron from oxygen.

$$O2 + e- \rightarrow O2$$

If oxygen loses two electrons, it is converted into hydrogen peroxide.

$$O2 + 2e^{-} + 2H+ \rightarrow H2O2$$

2.7.2 Superoxide:

Half of the oxygen molecule and hydroxyl radicals are the starters for reactive oxidation (Anurand and Boonme *et al.,* 1977). Lipids are per-oxidised with a radical of superoxide (Dahl and Richardson *et al.,* 1978). Perhydroxyl radical is formed by the addition of a proton to superoxide when pH levels are low (Yun-zhong Fang *et al.,* 2002).

2.7.3 Hydrogen peroxide:

Hydrogen peroxide is a major ROS which is important in oxidative stress. The iron molecule which is already present reacts with hydrogen peroxide to form hydroxyl radical. The hydroxyl radicals are more reactive (Fenton reaction). Hydrogen peroxide is not particularly sensible but an excellent oxidizing agent. In the presence of metal ions, it releases hydroxyl radicals. Usually, in living organisms, hydrogen peroxide is formed by two superoxide molecules. The regular metabolism forms these superoxide molecules.

$$2O_2^{\cdot-} + 2H+ \xrightarrow{SOD} H_2O_2 + O_2 \quad \text{(dismutase reaction)}$$

In this process, the primary by-product is oxygen.

Hydrogen peroxide is ROS because of the presence of oxygen free radical and derivatives of non-radical oxygen. It helps in releasing oxygen radical. It is essential in releasing hydroxyl radical, which damages the free radical of oxygen.

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \text{ (Fentons reaction)}$$

$$O_2^- + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2 \text{ (Haber-weiss reaction)}$$

$$O_2^- + Fe^{3+} \Leftrightarrow Fe^{2+} + O_2$$

$$O_2^- + Cu^{2+} \Leftrightarrow Cu^{+} + O_2$$

Then the oxidized forms of Ferric ($Fe3^+$) and cupric ($Cu2^+$) ions are the ferrous ($Fe2^+$) and cuprous (Cu^+) ions respectively which will react efficiently with hydrogen peroxide. The hydrogen peroxide and superoxide were taken out entirely in the absence of metal catalysts (Yun-zhong Fang *et al.,* 2002).

2.7.4 DPPH:

DPPH radicals will efficiently clean the natural compounds in the organism. In this activity, the antioxidants decrease stable DPPH to diphenyl picrylhydrazine which is yellow in colour. It is followed by reducing alcoholic DPPH to form DPPH-H (non-radical type). It is in the presence of an antioxidant that donates hydrogen, with this DPPH absorbance is reduced to 517nm. Hydrogen is donated by an antioxidant to form a potent DPPH molecule. It results by changing the colour to yellow. The radical form of DPPH has an absorbance capacity of 517nm which vanishes, followed by taking hydrogen radical or an electron from an antioxidant (Matthaus *et al.,* 2002). By high energy or transfer of electrons, the oxygen molecule is cleaved to highly reactive ROS in a stepwise manner. Biomolecules are formed by the pairing of ROS and free radicals. Due to this, the free radicals are formed in a continuous chain-like

manner. The freshly created free radicals, when bonded with one another, removing the free electrons from the outer orbital or make a bond with free radical scavenger (primary antioxidant), can stop the continuous chain-like formation of free radicals.

2.7.5 Nature of antioxidants:

Antioxidants counterbalanced free radicals.Antioxidants protect from the toxicity of Reactive Oxygen Species (ROS) in different ways are (a) stopping the formation of ROS (b) ROS changes the high reactive metabolites to less reactive ones to increase resistance (c) refilling the loss caused by ROS (d) giving a suitable atmosphere for the action of other antioxidants. The antioxidants complex in humans compensatesfor the non-enzymatic and enzymatic reactions to control the stress released by ROS. These kinds of antioxidants are of two kinds. They are endogenous and exogenous antioxidants. Endogenous antioxidants originated from the body and exogenous antioxidants are supplemented from outside to protect against the disease-causing organisms (Mittal *et al.,*1999).Endogenous antioxidants are the enzymes present inside the cells (as catalase, superoxide dismutase and glutathione peroxidase) which detoxifies the harmful metabolites released in the body (Reilly and Bulkley *et al.,*1990).

Superoxide anions catalyse for the formation of H_2O_2. The catalyst used is superoxide dismutase. The enzyme glutathione peroxidase reduces H_2O_2 (Delmaestro *et al.,* 1980). Oxidation of Glutathione and H_2O_2 or lipid peroxide reduction with glutathione peroxidase to form glutathione disulfide. The free radical scavengers' glutathione peroxidase, glutathione and superoxide dismutase are important for myocardial concentrations, which reduce hypoxia. This will enhance the occurrence of hypoxic cells at the injured place by free radicals formed by reoxygenation (Reimer and Jennings *et al.,* 1985).

Reactions catalyzed by endogenous antioxidant enzymes

$$2O_2^{\bullet-} + 2H^{\bullet} \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{\text{Glutathione peroxidase}} 2H_2O + GSSG$$

GSH-glutathione, GSSG- glutathione disulfide

As an overall view, the number of free radicals is controlled by endogenous antioxidants, which cause diseases. For controlling disease occurrence, antioxidant intake is mandatory in everyone's diet. These types of antioxidants are called exogenous antioxidants. Among them, vitamin E and C are significant, which are used to control many health issues.

Ascorbic acid:

Vitamin C or L-ascorbic acid is taken into our body throughdaily diet. It controls the free radicals efficiently and decreases damage caused by them. For the removal of free radicals from oxygen, an enzyme is not necessary. Ascorbic acid saves essential macromolecules from oxidative damage (Asada *et al.,* 1992). After reacting with tocopheroxyl, superoxide or hydrogen peroxide, it forms monodehydroascorbic acid. Reactions of superoxide with ascorbic acid:

$$2O^{\bullet} + 2H^{\bullet} + \text{Ascorbate} \longrightarrow 2H_2O_2 + \text{Dehydroascorbate}$$

The reaction with hydrogen peroxide is catalysed by ascorbate peroxidase:

$$H_2O_2 + 2\ \text{Ascorbate} \longrightarrow 2H_2O + 2\ \text{Monodehydroascorbate}$$

As an antioxidant, ascorbic acid again produces the antioxidants bonded with membranes like tocopherol, which removes peroxy radicals and one single oxygen molecule.

$$\text{Tocopheryl} + \text{Ascorbate} \longrightarrow \text{Tocopherol} + \text{Monodehydroascorbate}$$

Other antioxidants with lipid protection are carotenoids. The unpaired free electron is donated by superoxide to carotenoids. Carotenoid (β-carotene) acts as the best free radical scavenger. In the presence of more oxygen, β-carotene loses its antioxidant nature and acts as a pro-oxidant (Chandan *et al.,* 1995).

Synthetic antioxidants butylated hydroxyanisole; butylated hydroxytoluene clear the barrier generated by ROS. To control the side effects of synthetic antioxidants, the yield from herbs and consumable plants are targetednaturally, cells have a protective tendency for different diseases and microorganisms. Vegetables, fruits, phenolic

compounds and flavonoids from spices shield the cells from the loss by oxidative stress and increase our resistance to several vicious diseases.

2.7.6 Antioxidants and shielding from diseases:

According to public health studies, there is no link between the antioxidants present in the blood or tissue to the diseases that occur in the body. These health issues are leading to the death of the people. For this type of problem, antioxidants cannot act as extra immunity. We can increase antioxidants, vitamin E and Vitamin C in their body with daily routine food. (Vivekananthan *et al.,* 2003).

The need for antioxidants for Indians is utterly different from highly developed Western countries. For Indians, the antioxidants are supplemented through mixed vegetables, fruits, spices, curcumin and leafy vegetables grown in natural climatic conditions. In one way or the other, the minimum antioxidants are available.

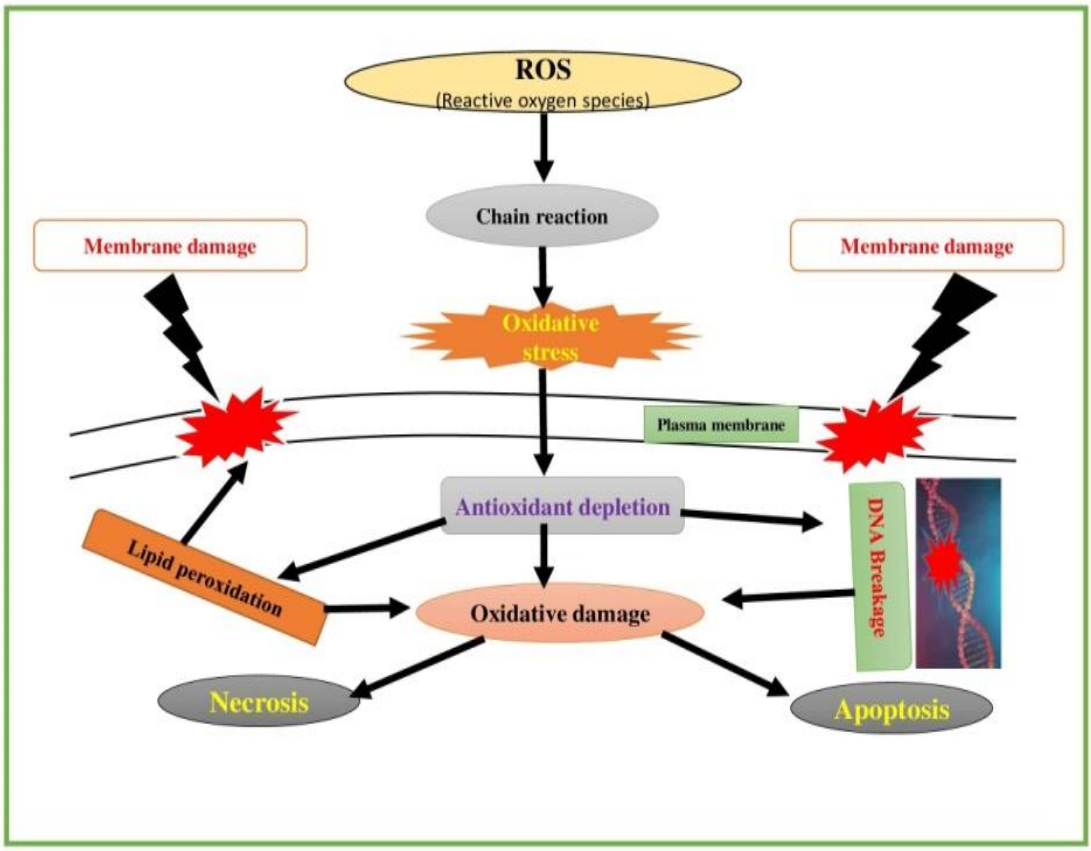


Fig. 13 flow chart of reactive oxygen species

2.7.7 Usage of ROS in cancer treatment:

ROS can control cell division and encourages cell destruction during cancer therapy. ROS mingles with some gene expressions and the pathways carrying the information to control the cancer cell growth (Thannickal & Fanburg, *et al.,* 2000). From their origin, they are oxidants that change the protein structure and its identity by free radicals. It controls the kinase (growth factor) and the pathway of phosphoinositide-3-kinase (Datta *et al*., 2000). ROS produced by cancer treating agents kill the diseased cells.

2.7.8 New variety treatment with antioxidants:

In all these years, by using antioxidants, we can easily control many diseases like Parkinson's syndrome, Alzheimer's, ageing, cancer and many more. But, with free radicals more efficiently, we can control heart stroke, diabetes, neurodegenerative disease, atherosclerosis and cancer.

Table.8 The following plants are reported to have antioxidant activity.

Plant name	Part reported
Acacia catechu	Bark
Acanthus ilicifolius	Leaves
Aconitum	Bark
Heterophylum	Rhizomes
Acoruscalamus	Roots & Leaves
Alchornealaxiflora	Aerial parts, Roots
Allium sativum	Aerial parts
Allium vienale	Leaf gel
Aloe vera	Roots & Rhizomes
Alpinia sp.	Std Extracts
Anthriscus cerefolium	Essential oil
Artemisia abyssinica	Essential oil
Artemisia afra	Entire plant
Artemisia apiaceae	Roots
Asparagus racemosus	Entire plant
Baccharis cordifolia	Stem, Leaves

Bacopa monnieri	Aerial parts
Ballaota acetabulosa	Aerial parts
Ballota	Entire plant
Pseudodictammus	Leaves
Boehmeria nivea	Bark
Brassica juncea	Entire plant
Burkeaafricana,	Aerial parts
Calamintha gladulosa	Entire plant
Calicotomevillosa	Lichen
Centaurea calcitrapa	Bark
Centella asiatica	Bark
Emblica officinalis	Fruits
Emilia sonchifolia	Leaves
Fagopyrum	Seeds
Esculentum	Bark
Ficus benghalensis	Root, Leaves
Garcinia atroviridis	Flowers
Glycyrrhiza glabra	Entire plant
Helichrysum	Seeds
Arenarium	Stem bark
Hemidesmus indicus	Entire plant
Hypericium	Leaves, Bark
Perforatum	Essential oil
Iberis amara	Essential oil
Lafoensiapacari	Aerial parts
Laminaria japonica	Entire plant
Mangifera indica	Fruits
Mentha piperita	Fruits
Momordica charantia	Entire plant
Murrayakoenigii	Wood oil
Nigella sativa	Aerial parts
Ocimum	Fruits
Ocimum	Entire plant
Ocimum sanctum	Root
Panax ginseng	Entire plant

Phyllanthus emblica	Anthocyanoside extract
Piper betle	Leaves
Prunus americana	Roots
Zingiber Sp	Rhizome

(Source: Plants with Antioxidant Activity CiddiVeeresham and KaleabAsres, *et al.*, 2005)

2.8 Phospholipase A_2:

Phospholipase A_2 belongs to the group esters. These enzymes can be seen in all human cells. They produce fatty acids and lysophospholipids from glycerophospholipids. PLA_2 proteins control the balance and disease development in the organs depend on their activation and controlling capacity of the inflammation. Based on the size, function, and need of different cofactors, 30 various Phospholipases A_2 (PLA_2) were discovered. All these were teamed into six groups. They are secretory PLA_2, platelet-activating factor acetylhydrolase, lysosomal PLA_2, Ca-independent PLA_2, cytosolic PLA_2 and adipose-specific PLA_2. Again, each group is divided into subgroups based on inhibiting capacity (Dennis E A *et al*. 2011).

2.8.1 The basic concept of PLA_2

PLA_2 has a significant role in forming eicosanoids of inflammations from the arachidonic acid pathway. The medicinal compounds act as a PLA_2 controller and act as an essential medicine for controlling inflammation in any part of the body (Dennis E A *et al*. 2011, & Magrioti V *et al*. 2010).

2.8.2 Secretory PLA_2

Six to eight disulphide bonds are present in the structure of secretory PLA_2. Crystallization occurs with a calcium cofactor at the enzymatic groove of histidine or aspartate. To control the bonding with monomers, the secretory PLA_2 reaction, as a result, enhances its aggregation with substrates. This concept is called a collection of interfacial substrates. Phospholipid membrane and secretory PLA_2 can quickly join by hydrophobic and electrostatic interactions. Different amino acids join based on their working. E.g., Tryptophan. This group has ten different subtypes. Secretory

PLA_2 has several uses in the human body. It is a potent enzyme to inactivate gram+ve, gram-ve bacteria and acts against viral infections too. It inactivates the bacteria by entering through murein and degradesthe plasma membrane. Secretory PLA_2 acts against the viral organisms, which control the receptors of chemokine. The primary function of secretory PLA_2 is to control inflammation by leukotrienes and prostanoids. By breaking the arachidonic acidit converts into an eicosanoid compound, which is a natural bioactive compound. This enzyme initiates the mast cells, which produce histamine by acting on anaphylactic reactions and is non-allergic.

Secretory PLA_2 controls the disease and its origin also is swelling in one place like atherosclerosis, asthma, rheumatoid arthritis, acute respiratory distress syndrome (ARDS) ulcerative colitis, Crohn’s disease and tumor cell growth. ARDS and asthma origin, development of these diseases and their effects have two different appliances compared to respiration. When secretory PLA_2 action is regular, it gives an increase in leukotrienes with chemokines' capacity and releases proinflammatory cytokines. In another way, in ARDS, the secretory PLA_2 abases the lung's outer layer with phosphatidylcholine and phosphatidylglycerol. It leads to swelling and stops the functioning of alveolar cells. The phospholipids hydrolyze in LDL (Low-density lipoprotein) particles,so the liquid accumulation is seen.

Secretory $PLA_2$is used for several different inflammatory diseases. It releases several inhibitory enzymes for asthma and several heart-related problems. It controls atherosclerosis marks and raises HDL (High-density lipoprotein) levels in mice. Secretory $PLA_2$is used for treating and preventing atherosclerosis. (Murakami, M., & Kudo I *et al*. 2002)

2.8.3 Cytosolic PLA_2

Cytosolic PLA_2 has six different structures, and each type has 749 different amino acids. Some of these amino acids have structures alike. This enzyme action at the phospholipid membrane was made easy by the calcium present in the cells for bonding. It acts on *the sn*-2 site in the arachidonic pathway and micelle substrates.

The action of cytosolic PLA_2 does not require calcium for its activity, like phospholipid membranes. By the growth of calcium levels in intracellular spaces, the cytosolic PLA_2 levels in the cell is affected.The calcium level makes the enzymes move naturally to the intracellular phospholipid membrane, present around the nucleus. The catalyst with intracellular calcium makes up for anionic molecules and increases the hydrophobic link with membrane substrates. It regulates the $cPLA_2$ function by phosphorylation. By hydrolysis of arachidonic acid, cyclooxygenase (COX) or lipoxygenase (LOX) are produced and regulate the cell cycle. The immunity gets increased by releasing eicosanoids. Cytosolic PLA_2 revitalizes the enzyme NADPH oxidase, which boosts immunity to control foreign organisms, causing infections.When the general purpose of the cytosolic PLA_2 is altered, it then opposes the swelling caused by organisms. The infections maybe rheumatoid arthritis, inflammation of the liver, acute respiratory distress syndrome and anaphylaxis. Cytosolic PLA_2 controls the various cancers caused in different parts of the human body and this action of the enzyme acts excess on the disease-causing organisms. Cytosolic PLA_2 enzyme is significant in controlling and making medicines for different inflammations like hyperalgesia and rheumatoid arthritis. When these are used on humans as experiments, safety measures have to be taken. Further studies have to be done quick for using this enzyme in treating different carcinomas and observe its cell cycle. (Leslie C. C. *et al.,* 2015)

2.8.4 Calcium independent PLA_2

Calcium independent PLA_2 is different from the other two types as calcium is not necessary for its activation (like cytosolic and secretory PLA_2). It is made up of 752 amino acids, which will control different functions as ATP adherence, caspase enzyme causing splitting and calmodulin interlink. It is the main enzyme for bringing about stability of a normal cell. It enhances the cell cycle occurrence and based on the cell functions, increases cell death. Ca-independent PLA_2 encourages the average production of bones, glucose-dependent insulin storage, and sperm cell maturity, regular work of smooth and skeletal muscles,also regain of nerves action after the injuries.Ca-independent PLA_2 participates in starting apoptosis in beta cells. It leads to initiate the disease and its symptoms in diabetes mellitus. These produce

superoxide compounds by lymphocytes, which leads to death. Ca-independent PLA_2 inhibitors are having less usage than other enzymes. It is used effectively with traditional chemotherapeutic agents for controlling ovarian related carcinomas. (Kudo, I., & Murakami, M *et al*., 2002)

2.8.5 Lysosomal PLA_2

Lysosomal PLA_2 gets its name because of its presence near the lysosomes in a cell. It has a three amino acid triplet which is catalytic. They are serine, aspartic acid and histidine, which makesacyl-transferase action with phospholipase. It is particular to phosphatidylethanolamine (PE) and phosphatidylcholine (PC) inside the lysosomes. In the absence of calcium, this enzyme acts singly. However, the action of this enzyme is controlled by calcium which is interconnected with other compounds. Lysosomal PLA_2degenerates phospholipids themselves. Degeneration of phospholipids occurs in the alveolar macrophages when they are in large quantities. With this, the number of phospholipids is controlled. Lysosomal PLA_2 converts lipid antigens to control the infections and uses CD1 proteins to represent leukocytes. *Mycobacterium tuberculosis* infects the pulmonary cells to form adaptive Th-1 immunity for T-cell. Lysosomal PLA_2 carries out atherogenesis and causesphospholipidosis. In the view of resistance, the less enzymatic function stops the pulmonary T-cell activation. The production of lysosomal PLA_2 decreases and it causes infection such as TB in pulmonary cells. An increase in mycobacterial cells shows less immunity for inflammations.

The disease caused by *Mycobacterium tuberculosis* activates pulmonary T-cells by Lysosomal PLA_2.We need to investigate the role of lysosomal PLA_2 action on other inflammatory conditions. (Fisher A. B.*et al.,* 2018)

2.8.6 Adipose-specific PLA_2

Adipose-specific PLA_2 shows calcium-free activity with PE (physical exam) and PC (post-meal) but does not show a reaction for acyltransferase. Enhanced quantity of arachidonic acid is produced with active enzymes and acts as a pioneer for prostaglandin-E. It results in the reduction of lipolysis and intercellular cAMP (cyclic

adenosine monophosphate) increase in enzyme action; the adipose activity increases by controlling intracellular cAMP. It is the tumour silencing agent in the adipocytes of humans. Adipose-specific PLA_2 enzyme controls the production of lipolysis andprostaglandin. By enhancing the protein, the synthesis of prostaglandin-E was more, cAMP level decreases and obesity or plumpness increases. The maximum number of tissues have this enzyme and has maximum enzyme level in adipocytic tissues. Their expression is also maximum in these tissues. This enzyme controls lipolysis, and the oxidation of fatty acids. It is the main enzyme causing obesity. When the adipose-specific PLA_2 enzyme levels are low, then the lifeless cells are more. If the enzymatic level increases, the oxidation of fatty acids in adipocytes is observed. These enzymatic actions are useful for treating heaviness or stoutness. (Yarla, N. S *et al.,* 2016)

2.8.7 Acetyl hydrolase as a platelet activator

It is the accumulation of enzymes, which act separately from calcium and have a triplet of amino acids having serine, aspartic acid and histidine. These make a bond with lipases and serine esterases. They react with lipoproteins present on the plasma membrane and initiates platelet-activating factor (PAF). PAF is a strong conciliator in controlling many diseases related to inflammations. (Glukhova A *et al.,* 2015).

The monocyte conversion into phagocytes is increased by the production of proteins with platelet-activating factor acetylhydrolase. To activate the LDL more enzymes are required with more catalysts. Initially, we used to think that it can control atherosclerosis. Later on, it is understood that the protein alone could increase the chance of the occurrence of atherosclerosis and coronary artery disease.

Acetyl hydrolase, a platelet-activating factor, brings about atherogenesis in blood vessels. This enzyme is associated more with oxidized LDL inflammation and enhances the production of plaques of atherosclerosis. Finally, this gives the resistance to fight against atherosclerotic infection. With the increasing activity of platelet-activating factor, acetyl hydrolase leads to neonatal necrotizing enterocolitis

(NEC). With this, the infants are affected with intestinal necrosis. by an exogenous form of the enzyme.We can overcome the NEC disease.

The platelet-activating factor acetyl hydrolase controls the probability of acute myocardial problems. This enzyme can control efficiently coronary artery related problems. (Ames, P *et al.*, 2018)

2.8.8 Pathway of Arachidonic acid (A.A.)

According to WHO, the most dangerous disease is cancer, leading to an increase in the worldwide mortality rate by itself. The mortality rate is 8.2 million per annum, and new cases are registered are 14.1 million (W.S. Bernard *et al.*, 2014). According to the sources, the new cases may increase to 70% in recent years.

The collaboration of UN Non-communicable Diseases Interagency Taskforce (2014) and other adhering bodies of U.N. with IARC (International Agency for Research on Cancer) and World Health Organization (WHO), to form The Global Action Plan (2013-2020) in controlling diseases like cancer.

Their main aim is to research prevention and control of cancer, educate the people towards the existing facilities, carcinogenesis mechanism and leading causes of cancer in humans. All over the world, many people were working on cancer cell growth, its mechanism, different therapies and prevention (Block, K. I *et al.*, 2015).

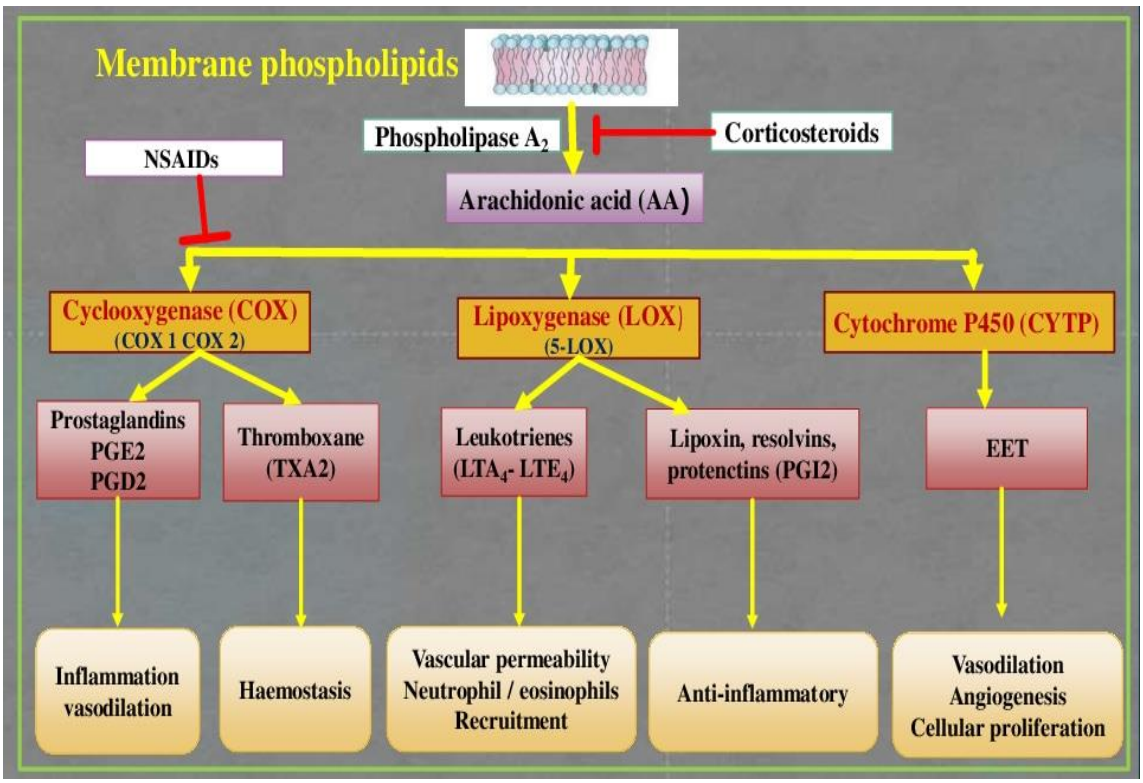


Fig. 14 flow chart of the arachidonic acid pathway

Initiation and development of cancer depends on several internal factors, such as growth-promoting factors and receptors like chemokines, cytokines, nuclear receptors, and transcription factors. Including all these, Arachidonic acid (A.A.) produced by lipid mediators plays an important role (Weber, D *et al*., 2010 Grizzi, F *et al*.2006). Apart from them, some external factors also induce cancer. They are smoking, some environmental factors, chemicals, dietary items, stress. They will activate the metabolites of A.A., a pro-tumour inducing factor. The essential metabolic pathway is the A.A. pathway, in which phospholipids and metabolites are involved. Lipoxygenases (LOXs), COX, PLA_2, prostanoids, leukotriene and many are involved. These play a significant role in the physiological functions A.A. pathway (Cuendet M *et al*.2000). The products and the enzymes that metabolise the A.A. pathway control the inflammation and regulate other organelle functions. It proved that the A.A. pathway plays the leading role in tumour formation and inflammation by clinical, cell-based and animal-based studies. COX has two isomers. The second isomer of COX is much expressive for

physiological as well as pathological areas, as in inflammation and tumorigenesis. Several investigators studied the inhibition of the A.A. pathway by natural or artificial inhibitors. The artificial inhibitors, produced from chemopreventive agents control swelling which helps in treating cancer patients(Smalley, W. E *et al*.1997). It is not prescriptible of having some adverse effects (Sinha, M *et al*., 2013). In this situation, there was hope on A.A., produced naturally and not having any side effects, when used as a therapeutic agent (Kim, Y. S *et al*.2009, Pan, M. H *et al*.2009, Samadi, A. K*et al*. 2015).

2.8.9 Steps involved in the Arachidonic Acid pathway:

Metabolites of Phospholipax A_2s

In the A.A. pathway, the first enzymes are PLA_2s. These PLA_2s can hydrolyse the phospholipids, which are attached to the membrane and having sn-2 acyl bonds. After this hydrolysis, AA, LPLs and free fatty acids were formed. We have identified more than 30 PLA_2 isomers based on function, structure, action mechanism, and distribution (Dennis, E. A *et al*. 2011). The isomers of PLA_2s are divided into families based on distribution, enzymatic properties, calcium requirement, substrate specificity and chemical structure as cytosolic-PLA_2 ($cPLA_2$), adipose-specific PLA_2 ($AdPLA_2$) and many forms. Based on invention, structure, and regular work PLA_2 family have groups, and subgroups. The first PLA_2s discovered are of secretory PLA_2s, present in different parts of the human body as pancreatic juices, fluid in the joints (synovial fluid), and other body tissues. It needs calcium for its catalytic activity.

$sPLA_2$ groups were divided based on a change in structure, discovery time and disulphide bond pattern into GI., GII, GIII, GV., GX. and GXII. These groups of $sPLA_2$are present in different parts and carry out different functions in humans. GIB of $sPLA_2$ present in the tissue of the pancreas carries out proper digestion of dietary phospholipids. GIIA is seen in the fluids of joints and forms tears (Qu, X. D*et al*., 1998). GIII, GV and GX andcollectively involved in increasing the resistance to viral and bacterial organisms (Mitsuishi, Met al., 2006). GV also increases resistance to fungal infections (Balestrieri, B *et al.,* 2009, Boilard E *et*

al.,2010) by forming many phagocytes in the blood. GXIIB is seen in the small intestine, kidneys and liver and is free for catalytic activity. Several other studies proved the anti-coagulant nature of $sPLA_2$ (Nevalainen, T. J *et al*., 2000). Because of the extracellular enzymatic activity of $sPLA_{2,}$it binds to specific receptors (PLA_2Rs) for signal transferring (Yu, J. A*et al*., 2012). Cytosolic PLA_2s (also called intracellular PLA_2s or GIV $cPLA_2s$) need calcium, so they are called calcium-dependent PLA_2s, divided into six subgroups (GIV A to GIV F). All these subgroups of GIV$cPLA_2s$ are activated with the rise of levels of calcium present in between the cells. (Dennis EA*et al.,* 1994). They are substrate specific to calcium ions, translocation of GIV$cPLA_2$ to a peri-nuclear membrane by the interaction of Asp53 and 93 with residues of C2 domain, converts arachidonic phospholipids(membrane-bound) to form LPLs and A.A. GIVA $cPLA_2$ Substrate selectivity took place by the dyads of Ser 228-Asp 549 in X-ray crystallography, which will help out in finding the cancers and inflammations. So, GIVA $cPLA_2$ is essential in the therapeutic and diagnosis of several cancers (Sastry Yarla, N *et al*., 2016). Some studies say that Ca+2 is not required as catalysts for $iPLA_2$ or GVI PLA_{2s}. Subgroups of GVI(A-F) $PLA_{2s}$are present in different organs of human beings, and their functions differ based on the cell and tissue. The life, growth and lysis of the cell depends on the constitutive enzyme released by GVIA PLA_2. PAF-AH is of GVIIA, GVIIB, GVIIIA and GVIIIB, which forms acetate and lysoPAF. So, PAF-AH initiates the development of the brain, homeostasis maintaining and acts against oxidative stress. GVIIA PLA_2 activates lipoprotein to react with glycerol-phosphorylcholine to form A.A. Thus we can call this $PLA_{2,}$ lipoprotein-associated PLA_2 (Lp- PLA_{2s}). In lysosomes, GXV or $LPLA_2$ is present, making the degradation of phospholipids. In adipose tissues, GXVI PLA_2 is present in large quantities. It is a calcium-independent PLA_2. Lipolysis inhibition of adipose tissue is catalysed by Ad PLA_2 and releases A.A. (Park J B *et al.,* 2012).

2.8.10 PLA_2 and inflammation:

PLA_2 work individually on inflammation-related defects like sepsis, arthritis, asthma and Adult Respiratory Distress Syndrome (ARDS). For people with arthritis, the

specific catalytic activity of PLA_2 is seen in lubricating fluid stored by the membrane lining joints and tendon sheaths. In them, a common enzyme is G-II of $sPLA_2$. Swelling in rheumatoid arthritis is the indication of elevation in the action of PLA_2 (Seilhamer J J *et al*., 1989). An inadequate amount of G II and G V groups of $sPLA_2$ in mice shows the low intensity of Arthritis. In some animals, the GIIA of $sPLA_2$ enhances the swelling activity in autoimmune Arthritis and is controlled by GV of $sPLA_2$. GIV of $sPLA_2$ participates in the production of eicosanoids used to treat many varieties of redness. The mice lacked in GIV of $cPLA_2$ pertinent to Arthritis produced by connective tissue. A low level of GX $sPLA_2$ leads to less production of eicosanoids. In the colorectal problem of Crohn's disease and inflammatory bowel disease, GIIA of $sPLA_2$ levels is more. In sclerotic patients, the PLA_2 level and its activity are below average. $sPLA_2$ levels will increase in proinflammatory and infestation but having low levels in pancreatic infections (Groeneveld A B *et al*.,1997). GII $sPLA_2$ gene is made inactive to reduce the effect of pancreatitis. More activeness or increase in the number of $sPLA_2$ cause Acute Respiratory Distress Syndrome (ARDS) (Touqui L *et al*., 2003). Recombinant mice with the gene GV $sPLA_2$ can efficiently control surface phospholipids which cause lung infection. The recombinant mice lack GIIA and GX $sPLA_2$ genes. GIIA, GV and GX $sPLA_2$ can efficiently manage and treat ARDS infection.

The recombinant gene of GX and the high appearance of GIIA of $sPLA_2$ easily control the bronchoalveolar and asthma infections (Triggiani M *et al*., 2009). By removing the GV, the PLA_2 gene reduces pulmonary disease and closes the air passage of hyper-responsiveness. With another inflammatory arbitrator, $sPLA_2$s synthesise mast cells when IgE is produced in response to a perceived threat. From the macrophages of lung $cPLA_2$, glucuronidase and mediators of inflammation $sPLA_2$ is released from neutrophils, elastase was formed (Ewa P *et al*., 2013). Another inflammatory infection is atherosclerosis. It is controlled efficiently by GIIA, GV and GX of $sPLA_2$. With the increase in the appearance of GIIA, PLA_2 leads to a rise in atherosclerotic lesions in recombinant mice. An essential function of GVIIA PLA_2 (also known as Lp-PLA_2) is increasing levels of atherosclerosis leading to heart-related problems. Lentiviral mediated RNAi can control efficiently Lp-PLA_2, which

regulates inflammation and atherosclerosis efficiently. Hence, Lp-PLA_2 treats patients with heart-related issues and atherosclerotic diseases (Steen, D. L *et al*., 2013).

2.8.11 PLA_2 and Cancer

PLA_2s can efficiently control cancer originating cells. In the $sPLA_2$ (GIIA, GIII and GX) and cPLA2 (GIVA) can control the tumors and inflammations. These two types of PLA_2s are more in the tumors of cancers. GIIA $sPLA_2$ enzymes were more in quantity in the patients with epithelial cancer, adenocarcinoma and breast cancer (Matsuda Y *et al.,* 1991). The moving cancer cells have more GII $PLA_2$and mRNA levels. We can see the PLA_2 part in the growth of breast cancer cells (Yamashita S *et al*., 1994). Lung cancer cell growth was controlled efficiently by GII $sPLA_2$. GI PLA_2 cells activate MAP kinases to increase the number of pancreatic cancer cells. $sPLA_2$ cells are more in number in stem cells of lung cancer and colon cancer rather than in healthy cells. If the $sPLA_2$ cell number is decreased, then the occurrence of lung cancer and colon cancer can be controlled (Buhmeida A *et al*., 2009). GIIA $sPLA_2$ have different roles in humans. The increase in their number may lead to tumor occurrence in lung, breast and prostate cells. Their decreased number could control the intestinal and gastric tumor formation (Fijneman R J *et al*., 2009). The recombinant gene of GIVA of PLA_2 encourages tumor formation in the lungs, pancreas, prostate, brain, breast, and colons leading to cancers (Linkous A *et al*., 2009). GIVA $cPLA_2$ occurrence was more in lung cancer cells of A549 and H460. When GIV $cPLA_2$ number is less, it controls the urethane-induced lung cancer cells; glioblastoma tumor formation reduces colon tumors (Ilsley J N *et al.,* 2005). When GIVA $cPLA_2$s were less, growth was not seen for Lewis Lung Carcinoma (LLC). The macrophages present around the tumor take up the role of $cPLA_2$ in lung tumorigenesis.GIVA $cPLA_2$ was lacking endothelial cells that are imperfect in shifting and cannot produce a vascular system in mice (Linkous A G *et al*., 2010)

GIVA $cPLA_2$ participates in causing tumorigenesis, metastasis, extensive migration, angiogenesis and isefficiently used to produce the anti-carcinoma drug. GVI calcium-independent PLA_2 ($iPLA_2$) plays an essential enzyme in spreading the tumor to a new place in the body (McHowat *et al*., 2011). The $iPLA_2$ cannot change tumor growth,

but these were present in mice's breast cancer cells than in normal mice. Apart from this activity, this enzyme is used for the long life of the cell. There is no activity of carcinogenesis of lysosomal PLA_2, but it plays an active role in phospholipid metabolism. Another type of PLA_2, adipose-specific PLA_2 of GXVI, acts as a suppressor of the tumor.

2.8.12 Natural products act as an inhibitor of PLA_2 as anti-inflammatory and anticancer agents:

Productsthat act as inhibitors of PLA_2 are plants, marine organisms and microorganisms. These inhibitors act as anti-inflammatory and anticancer agents. The secondary metabolites used as inhibiting agents are terpenoids, flavonoids, tannins and alkaloids. One of the secondary metabolites of a plant is a flavonoid, which can control the mammals' PLA_2 action. Rutin controls GII $sPLA_2$s activity, acts as an anti-inflammatory and (Lindahl M *et al.*, 1997) it is present in maximum plants as a glycoside. Flavanone and its other forms can efficiently control inflammations and cancer-causing cells. The secondary metabolite, coumestans extracted from the plant *Ecliptaalba*, efficiently control the PLA_2 enzyme (Diogo LC *et al.*, 2009). Morello flavone, a flavonoid, controls $sPLA_2$ enzymes and inflammations too. Morello flavone acts as an anti-inflammatory and as an antioxidant to control oral inflammations (Gil B *et al.*, 1997). Natural flavonoid Silibinin isolated from *Silybum marianum* acts as an inhibitor of GIIA $sPLA_2$. It controls inflammations, as well as cancer.Silibinin, acts efficiently as an anticancer enzyme in inhibiting MDA-MB 468 and MCF-7 cells present in humans' breasts. It acts on estrogen-dependent and estrogen-independent cancer cells in humans (Tyagi AK *et al.*,2004). Ellagic acid obtained from *Casearia sylvestris* efficiently acts as a controlling agent for PLA_2, an anti-carcinogenic and anti-inflammatory agent (DaSilva SL *et al.*,2008). From *Aristolochia* species, an alkaloid extracted as aristolochic acid, which controls PLA_2 efficiently. It controls snake venom PLA_2 and changes to secondary structure, which is not harmful to the organism. Aristolochic acid is not used to make medicine because of its toxic nature. But it controls inflammations and cancer tendencies too. (Chandra V *et al.*, 2002, Mariappan S *et al.*, 2012). Piperine is an alkaloid extracted from *Piper longum* and *Piper nigrum*. It acts as a good anti-inflammatory agent and

controls the arachidonic acid pathway too. Even it controls many types of $sPLA_2$s and $cPLA_2$ (Sastry Yarla, N *et al.,* 2016). Piperine shows anti-inflammatory, anti-carcinogenic nature. Along with controlling PLA_2s, it can efficiently control the accumulation of bloodplatelets.

Terpenoid extracted from the marine sponge is called Manoalide. It acts as anti-inflammatory, anti-tumorigenic and efficiently controls PLA_2 action (Dorandeu, F *et al.,* 2002). One of the metabolites obtained from marine organisms is Scalaradial,which efficiently controls PLA_2 activity. This controlling activity controls inflammations. From Citrus plants, a flavanone is isolated named Hesperidin. It controls PLA_2 efficiently and as a result, controls inflammations. Hesperidin also acts as anti-carcinogenic (Al-Ashaal H A *et al.,* 2011). Rosmarinic acid acts against snake venom PLA_2 controller. It is obtained from Cordia, Verbenaceae. Rosmarinic acid efficiently controls inflammation on epidermal cells and cancerous cells (Ticli FK *et al.,* 2005).

2.8.13 Artificial controlling agents for PLA_2 acts against inflammations and cancer cell growth:

PLA_2s can efficiently manage inflammations and cancer cell growth. To satisfy the needs of human beings, these PLA_2s were synthesised artificially in the industries. They made $sPLA_2$ type of enzymes, which can efficiently control inflammation and cancer cell growth. In those PLA_2s, some can manage the PLA_2s of snake venom and some ketones are made to stop the action of PLA_2 (Davidson F F *et al.,* 1986). A trifluoromethyl ketone GIVA PLA_2 which is more effective on inflammations occurs on the skin (Bristol-Meyers Squibb). Bromo enol lactone (BEL) is equal to GVIA PLA_2 and can control prostate carcinoma cell growth by producing $iPLA_2$ suppressors (Sun B *et al.,* 2008). BEL suppresses the prostate and the starting of eGFR in carcinogenic cells of the pancreas. BEL can efficiently control the metastatic cell growth in ovarian epithelial cells. They developed enzymes that control the growth of cardiovascular inflammations and acute coronary syndrome. They could not give a remedy for myocardial infarction.

PLA_2s are a type of enzyme which are divided into groups and sub-groups. It is cosmopolitan in living organisms. All kinds of PLA_2s are essential for general functions. If anyone of it expresses majorly, then it may result in inflammations and being carcinogenic.

GIIA, GV, and GX sPLA_2s have significant control over inflammation and cell division to form the tumor. GIVA and $iPLA_2$ are involved in initiating inflammation and being a carcinogen, but $iPLA_2$ has some other uses in an organism's regular life. Lp-PLA_2 is not at all involved in carcinogenic issues. Lp-PLA_2 increases atherosclerosis, so it is necessary to develop controlling enzymes for this disease as Lp-PLA_2 inhibitors. Ad-PLA_2 is having one significant role in controlling tumor formation.

Based on these good qualities, PLA_2s are used in making medicines. But they are having different activities and more forms. Because of having various expressive characters, it is challenging to study and use in treatment. After the severe study, when the PLA_2 combined with the arachidonic acid pathway, it can be used as a medicine for different problems.

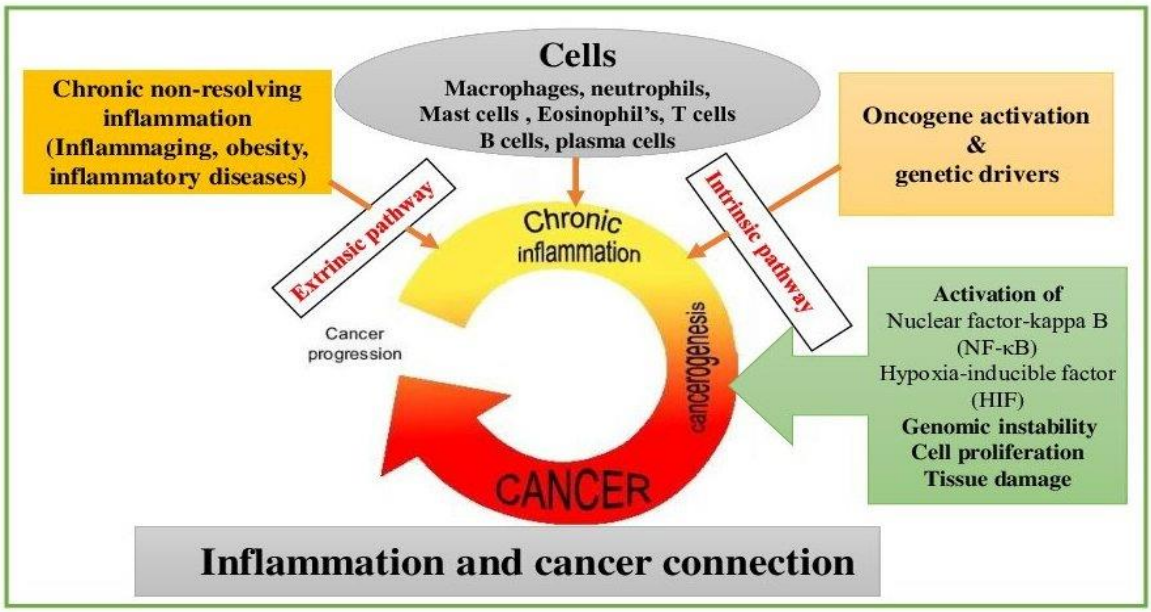


Fig. 15 path of inflammation and cancer connection

(**Inflammation and cancer connection**: When the problem is decreasing, that occurs with the change in the genotype or caused by any external means, results in itching and swelling. The cells present in the swelling and cancer area starts by forming abnormal white blood cells that start glowing; that area looks hot and scorching. Transcription factors NF-kB, HIF-1a and STAT-3, can change the occurrence of inflammations in cancers. This occurs with the change in the genotype, changing the enzyme formation by genes or by changing the gene expression at a specific movement, resistance to stop cell death, prevents the tumor formation and changing the shape of tissues to stop the entry of tumorcells **(supply: Porta et al., 2009, immunobiology)**

2.9 APOPTOSIS

The principle of apoptosis was given by German scientist Carl Vogt (1842). Walther Flemming (1885) explained in detail a programmed cell death. In 1972, John Foxton Ross Kerr, Andrew Wyllie and Alastair R. Currie gave the process of cell death in an article, which is called apoptosis. Apoptosis is a Greek word, means falling off or dropping off. It is related to the plants as leaves fall off or dropping of petals from flowers.

In the organism, once apoptosis starts, it won't stop. The apoptosis occurs during the development of organs, making the tissues and developing immunity support for the organism. Whereas in necrosis, lysis of cells occurs by unlimited cell death, inflammations and has some severe health issues (Leist and Jaattela *et al.*, 2001). Apoptosis removes unnecessary and non-adaptive cells. With apoptosis, the cell size decreases and dissociates into small fragments. The genetic material disintegrates. Altogether we can say apoptosis is a manageable and suitable method which is observed inner to the membrane (Raff & Martin, *et al.*,1998). Before the apoptotic cell membrane bursts, macrophages carry out phagocytosis (Raff & Martin, *et al.*, 1998). With this, inflammations never occur during apoptosis. Apoptosis is engaged in all metabolic activities and removes cells that are not useful. The immune system is having T-lymphocytes which removes or takes off impaired cells in the organism. These T-cells develop in the thymus and are checked before releasing into the blood

as they are active for the foreign antigen only. Apoptosis removes the T-cells acting on the normal cells,if the apoptosis is not acting correctly, it leads to viral infections, disorders in the immune system and cancer formation. When apoptosis occurs more, leads to AIDS, myocardial Ischemia and neurodegenerative disorder (Qiao and Wong, *et al.*, 2009).

Apoptosis changes the cells morphologically to show different functions and look different from other cells. Apoptotic cells decrease, distortion, genetic material condenses and lose contact with other cells. The plasma membrane shows swelling, so at last, the cell busted into small fragments as caspases (Trump *et al*., 1997). The caspases have the only cytosol and are removed by macrophages without any swelling.

2.9.1 Different pathways of apoptosis:

Apoptosis starts from the external or internal lysis of cells; the external lysis starts from outside the membrane having Fas receptors which act as receptors for death. The internal lysis starts with the factors produced by mitochondria.

2.9.1.1 The External or TheExtrinsic Pathway:

Ligands (also called signal molecules) are delivered from other cells during the extrinsic pathway. On a specific cell, these ligands are attached totransmembrane death receptors (Fas receptors) causing apoptosis. The standard killer cells of the immune system present outside are known as Fas-ligand (FasL) (Csipo *et al*., 1998). On the infected cell, the Fas receptors (death receptor) are attached by FasL. They activate many other receptors external to the cell leading to form Fas-Associated Death Domain protein (FADD). This FADD is present on the inner side of the receptors, facing towards the cytoplasm. FADD produces an initiator protein caspase-8, which makes a complex called Death Inducing Signal Complex (DISC). The degeneration of the cell is activated when caspase-3 is initiated by caspase-8. The initiated caspase-8 splits a protein called BID to tBID. The tBID activates the mitochondrial membrane to produce cyt C, which is used for the internal lysis of the cell. (Adrain *et al*., 2002).

2.9.1.2 Internal cell lysis or The Intrinsic pathway:

The stress leads to the intrinsic pathway. Mitochondria may cause stress because of the changes in mitochondria occurred by damaged DNA (Adrain *et al.*, 2002). Once the signals are released, the BAX and BID proteins produced before the cell death attach to signals present on the mitochondrial outer membrane. It liberates the internal contents. The BAX and a BID signal cannot initialize the complete release. One more pro-apoptotic protein present inner to the mitochondria is BAK. It also initiates the production of cytochrome C and contents present on the cristae of mitochondria (Hague *et al*., 2004). In the cytoplasm, cytochrome C combines with ATP and Apaf-1to form a complex. Caspase-9 initiated with this complex is a protein initiator. Apoptosome is formed from the complex of cyt C, ATP Apaf 1 and caspase 9. The apoptosome initiates a protein called caspase-3, which promotes the destruction of cells. From the mitochondria, cytochrome C and element initiating produced for degradation of DNA and Smac/Diablo proteins. These proteins will control the apoptosis controlling proteins (Hague *et al*., 2004).

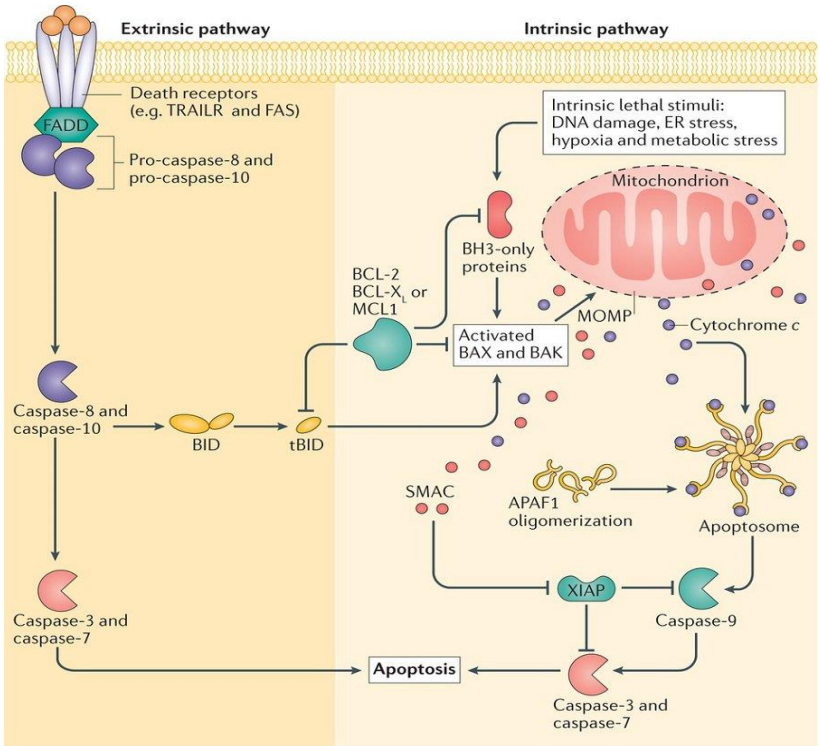
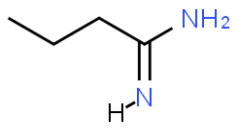
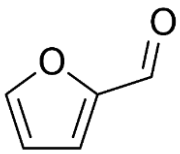
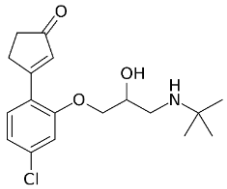
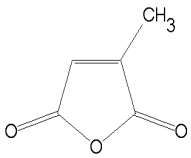
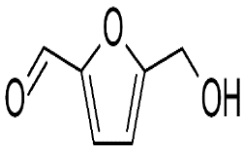
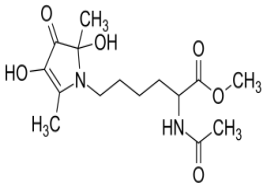
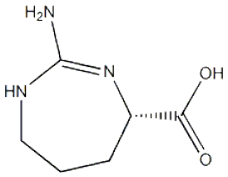
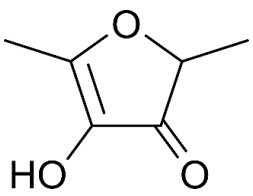
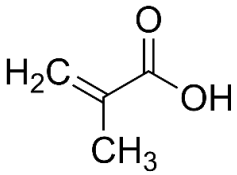
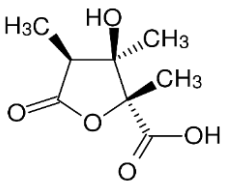
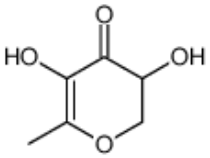
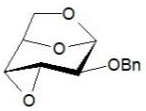
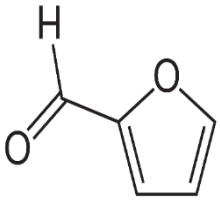
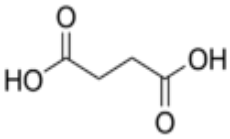
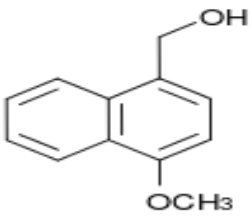
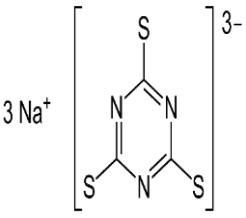


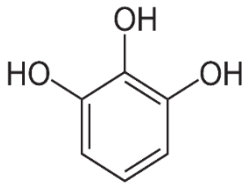
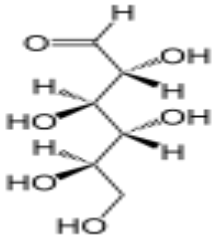
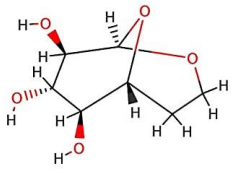
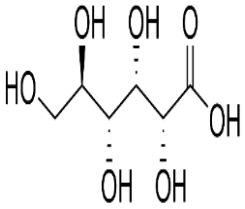
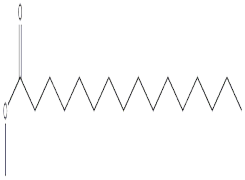
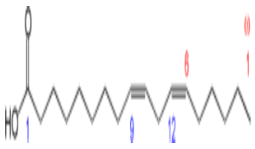
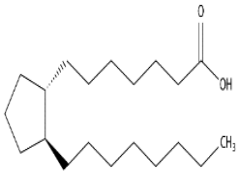
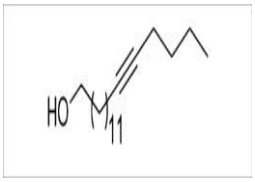
Photo reference: (Biologydictionary.net Editors. (2017, June 06). Apoptosis. Retrieved from https://biologydictionary.net/apoptosis/)

Fig. 16 Apoptosis life cycle

2.10 Triphala Plant's Constituents:

Chemical constituents of *Phyllanthus emblica*

1 	**2** 	**3** 	**4** 
5 	**6** 	**7** 	**8** 
9 	**10** 	**11** 	**12** 
13 	**14** 	**15** 	**16** 

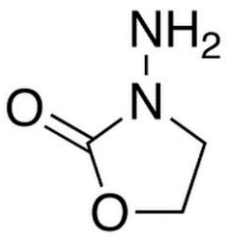
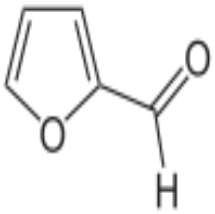
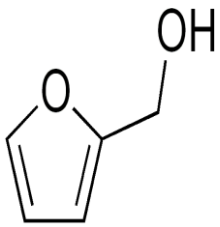
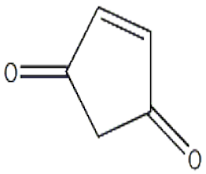
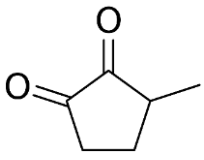
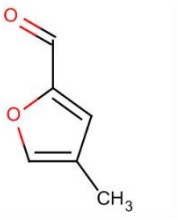
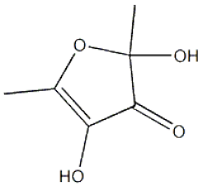
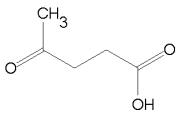
17 	**18** 	**19** 	**20** 
21 	**22** 	**23** 	**24** 
25 	**26** 	**27** 	

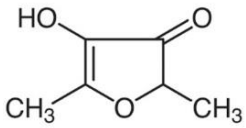
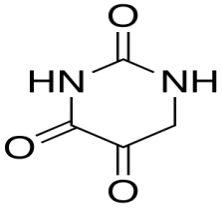
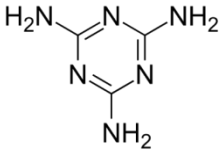
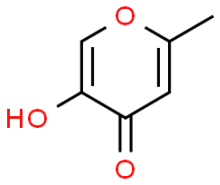
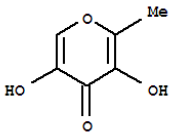
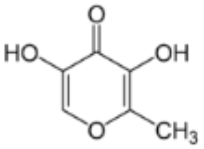
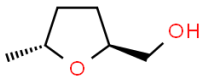
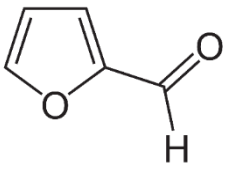
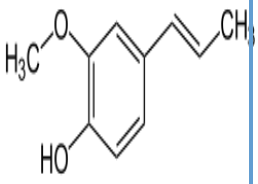
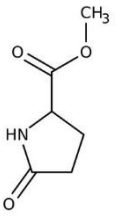
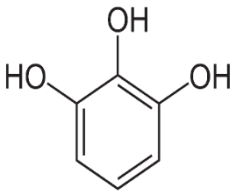
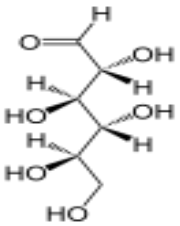
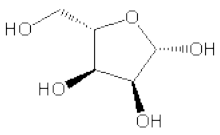
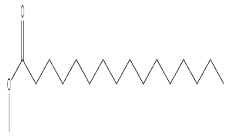
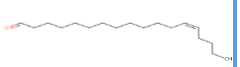
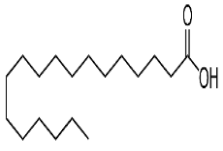
Source: Chemical constituents of *Phyllanthus emblica* (V.eugin amala 2016)

S. No	Compound Name	S. No	Compound Name
1	Butanediamide	15	Undecanol-5
2	Furfural	16	2,4(1H,3H)-Pyrimidinedione, 6-(hydroxymethyl)-1,3 dimethyl
3	2-Cyclopentane-1-one,2-hydroxy	17	1,2,3-Benzenetriol
4	2,5-Furandione,3-methyl	18	D-Allose
5	2-Furancarboxaldehyde, 5-methyl-	19	1,6-Anhydro-beta-D-glucofuranose
6	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	20	Gluconic acid

7	1H-1,3-Diazepine, 4,5,6,7-tetrahydro-2-methyl-	21	n-hexadecanoic acid, methyl ester
8	2,5-Dimethyl-4-hydroxy-3(2H)-	22	n-Hexadecenoic acid
9	Acrylic acid, 3-amino-3-cyano methyl ester	23	9,12-Octadecadienoic acid,
10	5-Oxotetrahydrofuran-2-carboxylic acid, methyl ester	24	7-Hexadecenoic acid, methyl
11	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	25	Octadecanoic acid, methyl ester
12	1,6,3,4-Dianhydro-2-deoxy-beta-d- ribo-hexopyranose.	26	13-Octadecenal, (Z)-
13	2, Furancarboxaldehyde,5	27	Octadecanoic acid
14	Butanedioic acid, hydroxy-, (S)-		

Chemical constituents of *Terminalia bellirica*

1 	**2** 	**3** 	4 
5 	**6** 	**7** 	**8** 

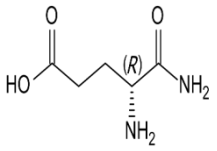
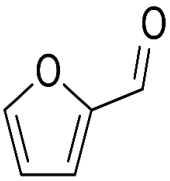
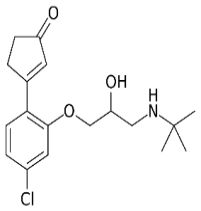
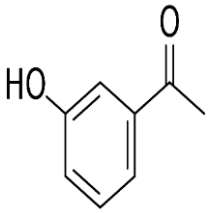
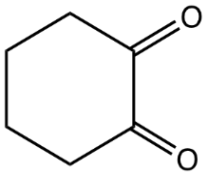
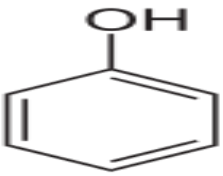
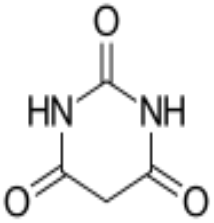
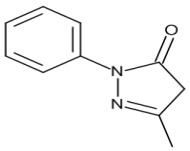
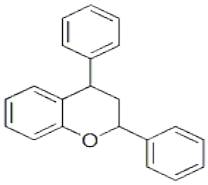
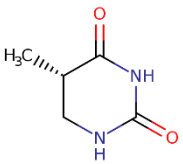
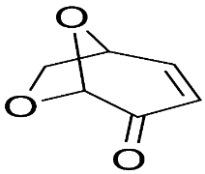
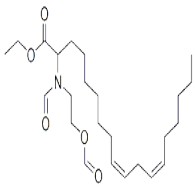
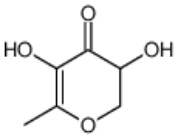
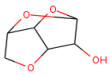
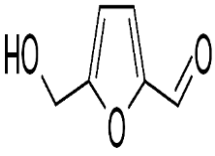
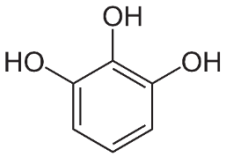
9 	**10** 	**11** 	**12** 
13 	**14** 	**15** 	**16** 
17 	**18** 	**19** 	**20** 
21 	**22** 	**23** 	**24** 
25 	**26** 		

Source: Chemical constituents of *Terminalia bellirica* (V. eugin amala, 2016)

Chemical constituents of *Terminalia bellirica*

S. No	Compound Name	S. No	Compound Name
1	3-Amino-2-oxazolidinone	14	4H-Pyran-4-one, 3,5 dihydroxy-2-methyl-
2	Furfural	15	2-Furanmethanol, tetrahydro-5-methyl-, trans-
3	2-Furanmethanol	16	Furan carboxaldehyde, 5-(hydroxymethyl)-
4	2-Cyclopentene-1,4-dione	17	Phenol, 2-methoxy-4-(1propenyl)-
5	1,2-Cyclopentanedione	18	DL Proline, 5oxo, methyl ester
6	2 Furancarboxaldehye 5-methyl-	19	1,2,3-Benzenetriol
7	2,4-Dihydroxy-2,5-dimethyl-3(2H)-	20	D-Allose
8	Pentatonic acid, 4-oxo-	21	1,6-Anhydro-a-d-galactofuranose
9	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	22	Tetradecanoic acid
10	2,4,5-Trihydroxypyrimidine	23	Hexadecanoic acid, methyl ester
11	1,3,5-Triazine-2,4,6 triamine	24	n-Hexadecanoic acid
12	4H-Pyran-4-one, 5-hydroxy-2-methyl-	25	13-Octadecenal, (Z)-
13	4H-Pyran-4-one, 2,3-dihydro 3,5-dihydroxy-6-methyl-	26	Octadecanoic acid

Chemical constituents of *Terminalia chebula*

1 	**2** 	**3** 	4 
5 	**6** 	**7** 	**8** 
9 	**10** 	**11** 	**12** 
13 	**14** 	**15** 	**16** 

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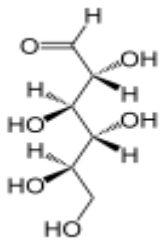		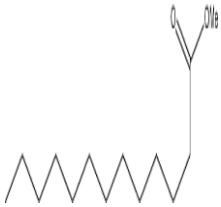	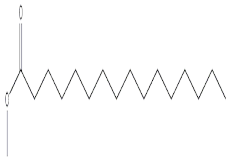
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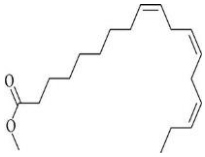
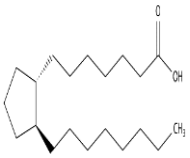
			

Source: Chemical constituents of *Terminalia chebula* (V. eugi amala 2016)

Figure.17 Different compounds of Triphala Plants ligands

Chemical constituents of *Terminalia chebula*

S. No	Compound Name	S. No	Compound Name
1	Iso glutamine	13	4H-Pyran-4-one, 2,3-dihydro-
2	Furfural	14	1,4:3,6-Dianhydro-a-d-glucopyranose
3	2-Cyclopentene-1-one, 2-hydroxy	15	2- Furancarboxaldehyde
4	Ethane one, 1-(3-ethyloxiranyl)-	16	1,2,3-Benzenetriol
5	1,2-Cyclohexanedione	17	D-Allose
6	Phenol	18	Tetradecanoic acid
7	2(1H)-Pyridinone, 6-hydroxy-	19	Tridecanoic acid, methyl ester
8	3H-Pyrazol-3-one, 2,4-dihydro-2,4,5-	20	n-Hexadecanoic acid
9	5H-1,4-Doxepin, 2,3-dihydro-2,5-dimethyl	21	9-Octadecanoic acid (Z)-, methyl ester
10	Hydro uracil, 1-methyl-	22	Octadecanoic acid, methyl ester

11	Levoglucosenone	23	13-Octadecenal, (Z)-
12	5-Formyl-3-methyluracil	24	Octadecanoic acid

2.11 Morin:

Morin, a pentahydroxy flavone, is an essential compound in many Moraceae plants. Morin (2',3',4',5,7– pentahydroxyflavone) contains 3 phenolic rings. It is a C15 flavonoid structure. ($C_{15}H_{10}O_7$) Usually, the flavonoids have flexible binding capacity.

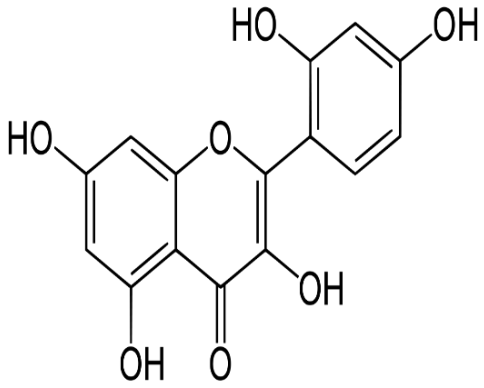


Figure 19 Chemical structure of morin

2.11.1 Natural sources of Morin:

In the vegetative parts of many plants, a high quantity of Morin is found. List of the different natural sources of Morin:

Psidium guajava (Guava):

Guava is available in different varieties all over the Globe. The common species is *Psidium guajava*. In the leaves of guava, Morin is present in high quantity. Other forms of Morin are also found as Morin-3-O-arabinoside, Quercetin and (Rattanachaikunsopon, P.*et al.,* 2007).

Prunus dulcis (Almond)

The type of fruit it has is Drupe; the hull is rich in Morin (Takeoka, G. R *et al.,* 2003). Almonds are distributed widely in North Africa, the Middle East and in the Sub continent of India.

Chlorophora tinctoria (Old fustic)

This plant is seen in South America. We can extract a Yellow coloured pigment rich in Morin. All the plant's vegetative parts, i.e., bark, leaves, fruit, and other vegetative parts, contain Morin in abundant quantity. Initially, military people used this for colouring; now, it is used for the medicinal purpose (Lamounier K.C.*et al.*, 2012).

Acridocarpusorientalis:

It comes under the family Malpighiaceae, where the plant parts contain the flavonoid Morin in leaves, fruits and bark. This is in yellow, which is phytochemical in nature. These plants are seen mostly in tropical and subtropical regions of Africa. (Hussain, J*et al.*, 2014)

Maclurapomifera [Osage orange]

This is a tree or shrub, which is deciduous. The fruit is rich in Morin, which attains yellowish-green colour after ripening. All the plant parts and fruits contain phytochemical Morin in large quantity [Tsao R *et al.*, 2003.].

Allium cepa (Onion)

Morin can be extracted from the onion peel, which protects DNA from exposure to toxic substances. The Morin derived from this is having therapeutic potency (Majidi, S. M., & Hadjmohammadi, M. R.*et al.*, 2019)

Malus pumila (Apple)

According to recent studies, Apple is the flavonoid fruit in Americans' daily diet and in other countries. Morin is present in more quantities in the peel of the fruit.

Beverages:

Tea (*Camellia sinensis*): Green tea has plentiful different flavonoids, anti-inflammatory and anti-oxidant by nature. It is having many phytochemicals, in that Morin is one of the important phytochemicals. All the benefits from the health point of view come with Morin associated with or without other phytochemicals in it. (Prasanth, M. I *et al.*, 2019)

Coffee:

The most common source of Morin in our daily life is Coffee. Morin coming from Coffee acts against Cancer causing cells and controls oral cancers in vitro and in vivo.

Red wine

A high quantity of flavonoids and Morin is found in red wine than in any other wine. This is having health benefits as anti-inflammatory, anti-oxidant and anti-apoptotic. With other phytochemicals, Morin increases the flavour and taste of red wine. Because of this, this is accepted as a recreational drink and good for health for both males and females(Choudhury, A *et al.,* 2017).

Cereal grains

The cereals have different flavonoids with different phytochemicals like Morin, suitable for health and maintaining a good appetite. (Choudhury, A *et al.,* 2017)

2.11.2 Metabolism of Morin in the body:

Morin is collected from natural products as derivatives of glycosylated products or phytochemicals (Subash S., Subramanian P *et al.,* 2009). When this was taken along with any food material, it reachesthe gut, then hydrolysed by converting into aglycone, which is taken by the stomach's membranes quickly. By the bacterial action on unabsorbed food in the large intestine, aglycone Morin is formed, which is transported through a specific tagged protein into the cellular membrane. This transportation needs energy. Many researchers proved that Morin has low permeability levels to pass through the intestine membranes (Oteiza P.I. *et al.,* 2005). Extrusion of Morin was done by Multidrug Resistance Associated Protein-I (MRAP-I) in the gut (Wesolowska O *et al.,* 2009). Morin uptake can be increased by the selective chemical components which inhibit the MRAP-I.

2.11.3 Therapeutic use of Morin

Morin, with its anti-oxidant nature, takes away the disease-causing particles. Morin's pathological conditions can be improved like hyperglycemia, insulin resistance, glucose intolerance [Caselli A *et al.*2016.]. Morin inhibits macrophage activities to

increase the health condition in a better way. By the injection of Morin, the TNFα levels are decreased, which causes several problems in the system (ChoudhuryA *et al.*,2017). Morin can control the cytokines inflammations IL-Iβ effectively. Like this, Morin can control many gene actions, which causes different problems in humans.

2.11.4 Therapeutic use of Morin in Alzheimer's disease:

Amyloid-beta causes Alzheimer's. β-secretase enzyme prepares amyloid-beta from Amyloid Antecedent Protein (APP) (Choudhury A *et al.,* 2017). The internal and external change occurred due to a change in redox balance, affecting the more sensitive neurons in nature. Changes for a long period may cause chronic inflammations, which turns into apoptotic mechanisms. By injecting Morin, it starts inhibiting the Glycogen Synthase Kinase-3β (GSK 3β), used as an enzyme during Alzheimer's disease pathology (Gong E *et al.,* 2011). By inhibiting the GSK 3 β, it can change cellular pathways. This impairs axonal transport, starts a mechanism for apoptosis, which changes the neurological spectrum.

2.11.5 Role of Morin in Parkinsonian pathology:

Theprogressive neurodegeneration is caused by redox imbalance and nigrostriatal neurodegeneration (Varma, D *et al.,* 2015). Morin can control the apoptosis in cell-line PC12 intoxicated with MPP+(1-methyl-4-phenylpyridinium ion), activation of caspase-3 and ROS formation (Zhang, Z. T *et al.,*2010). So we can say that Morin has a high capacity to save dopaminergic neurons from the neurotoxic effect of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine). Morin reduces the toxious enzyme release such as dopamine and serotonin(McDowell, K *et al.,* 2012)

2.11.6 Anti-cancer activity of Morin:

Now-a- days all over the world, the mortality rate increases with Cancer. This causes mainly when Oncogenes gets activated with a small change in the protein products or change in their pathways or apoptosis in the cells. This will change the cell towards uncontrollable division, growth and invasion. Among many natural photocomponents, Morin only controls the DNA's damage, making the pathways go in the right way (Kim, J, Park G, & Lee J.H, *et al.*,2008). Morin can control the

activity of carcinogenic cells in forming a mass of cells and can stop the formation of tumour-induced by chemical compounds. Morin can change the cells' pathways to control the carcinogenesis and make the cells at G2 or M phase for a more extended period of time. This stops the cell division from forming tumors (carcinogenesis) but cannot cause apoptosis.

Morin activates Bax and caspase-3, which activates caspase-9, which increases cell death in prostate cancer and Leukemia (HL-60) cells. Overall, this can increase the release of Cyt-c from mitochondria. The final result, by inducing Morin, increases the carcinogenic cell apoptosis by decreasing the non-apoptosis for Bcl-2. This Bcl-2 fastens apoptosis. Morin has a considerable capacity to make bonds with metal ions and creates complexes like zinc, iron, copper, vanadium, chromium and cobalt. Morin-metal ion complex is needed to fulfil the Haber-Weiss reaction, which removes the ROS complex from the cytosol.

2.129, 10 anthraquinone:

Anthraquinone**,** an isomer with various Quinone derivatives, refers to 9, 10 anthraquinone where keto groups are locked on the central ring for this aromatic organic compound having a formula of $C_{14}H_8O_2$. Anthraquinone is also called anthracenedione or dioxoanthracene, belonging to the Quinone family. It has there benzene rings.

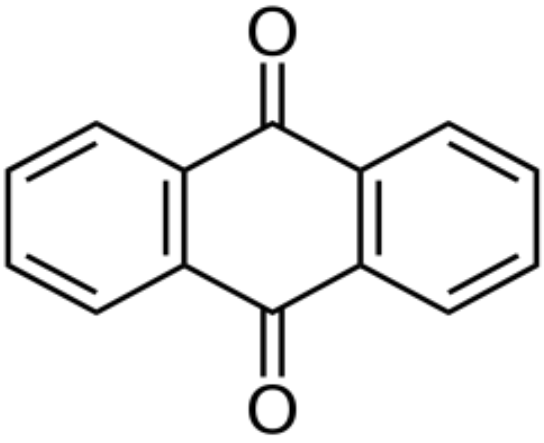


Figure 20. Chemical structure of 9,10 anthraquinone

Melting point: 286 °C **Formula:** $C_{14}H_8O_2$ **Density:** 1.31 g/cm³ **Boiling point:** 379.8 °C **Molar mass:** 208.216 $g.mol^{-1}$ Solubility in water-insoluble structure. This important class of natural and synthetic compounds has a wide range of medical applications as laxatives, diuretics, phytoestrogens, antiplatelet, anti-fungal, antiviral and anti-cancer properties. Anthraquinone has potential industrial use in manufacturing dyes, textile, food colourants, pulp industry, bugand bird repellent. Its inherent nature is further continuously probed as a dry template for cancer treatment.

2.12.1 Natural sources of 9, 10 anthraquinone:

A wide variety of species includes anthraquinone derivatives in higher plants (Caro *et al.,*2012). While these substances are distributed less in other phenolic molecules (e.g. flavonoids), in edible plants and vegetables. A type of anthraquinone is present in higher plants such as *Rumex, Rhamnus, Aloe* and *Cassia*. The structure is changed by having two hydroxyl groups at R1 and R8 positions in the aromatic rings.

Anthraquinone extracted in large quantity from the Rubiaceae family (e.g., *Rubia, Galium*, Moringa, etc.) is used for evaluation (Caro *et al*., 2012). Usually, anthraquinone bonded to sugars forms soluble glycosides (Teuscher and Lindequist 1994, Thomson 1997, Lu *et al.*, 1998, Derksen *et al*., 2003). Anthraquinone is present in the flowers as a laxative for a long time. The purgative tablets made from plants have different anthraquinone types as chrysophanol, aloe-emodin, emodin or rhein. Purgative drugs are used in small quantity for healing. Readily available purgative drugs cascara (from the bark of *Rhamnus purshiana*), frangula (from the bark of *Rhamnus frangula*) and senna (from the leaves and fruits of *Cassia angustifolia* and *C. senna*) used in making medicines. It is present in less quantity in vegetables (e.g., cabbage, beans, peas and lettuce), herbs and alcohols obtained from herbals. Anthraquinone in peas is (0.04 to 3.6 milligrams), cabbage & lettuce (5.9 milligrams) and beans (36 milligrams) (Mueller *et al.*, 1999). The alcohols obtained from herbs have less anthraquinone than 1 milligram/kilogram (dry weight).

CHAPTER - 3
MATERIALS AND METHODS

Dulbecco's Minimum Essential Medium Eagle (DMEM) and HPLC grade solvents were procured from Hi-Media Laboratories Pvt. Ltd, (USA). Fetal Bovine Serum (FBS) was procured from Cistron Laboratories (UK). Methyl Thiazolyl diphenyl Tetrazolium bromide (MTT), Trypsin, Dimethylformamide (DMF), Dimethyl Sulfoxide (DMSO), Sodium dodecyl sulphate (SDS) were bought from Sisco Research Laboratories. Chloroform ethylacetate, Methanol, n-Hexane and DCL were obtained from Fischer Scientific laboratories (USA). Hydrogen peroxide was obtained from Merck laboratories (UK). From Sigma-Aldrich labs (USA) purchased 2,2-diphenyl 1-picrylhydrazyl (DPPH).

3.1 Glass apparatus sterilization:

The glass apparatus used in the lab were first cleaned with soap water, washed thoroughly under running tap water. Before drying, these apparatuses were cleaned with distilled water. The glass apparatus and culture medium were sterilized in an autoclave for 20 min at 120°C and 15 lbs pressure.

3.2 Collection of Plant material:

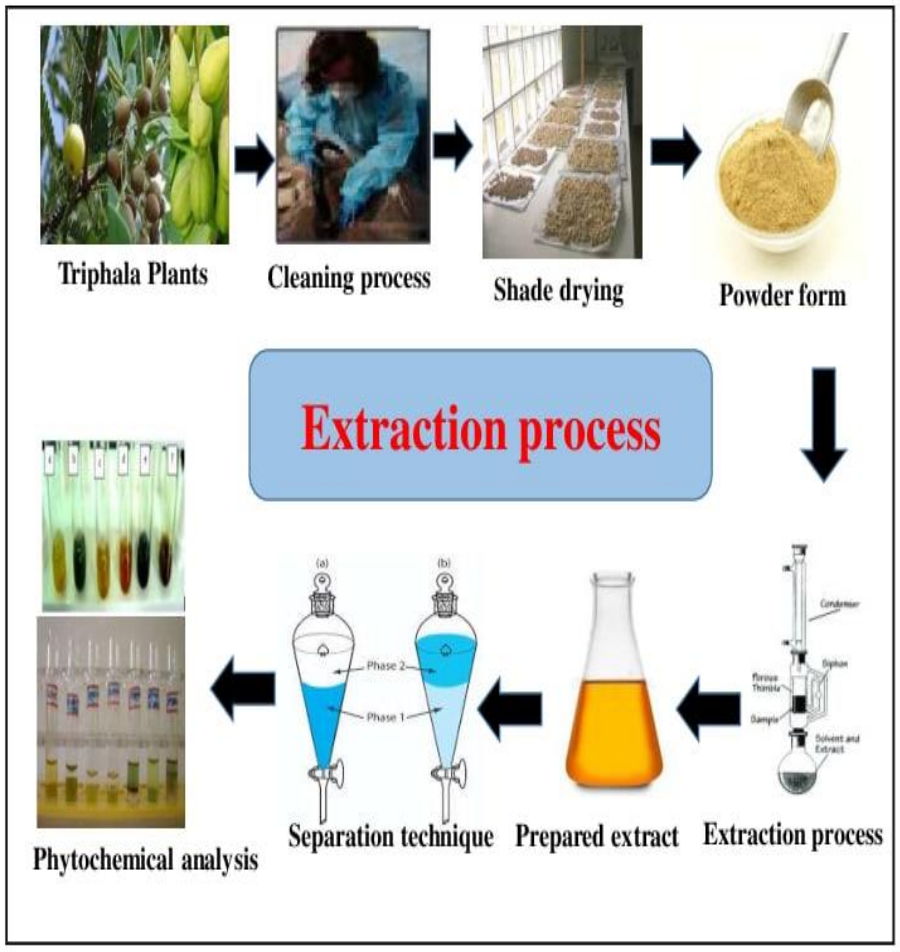
Fruits of *Terminalia chebula, Phyllanthus emblica, and Terminalia bellirica* were collected from Vikarabad Forest range, Vikarabad (Dist. Vikarabad), (Near Hyderabad) Telangana, India. The plant identification was done according to the Botanical Survey of India (BSI), Deccan Regional Centre, Hyderabad (**No: BSI/DRC/17-18/TECH/272**). The collected plant parts were appropriately dried, preserved in the herbarium sheets in the Botany lab of SAP Degree and PG College, Vikarabad (Dist. Vikarabad), Telangana. The voucher numbers are SAP/201/2017, SAP/202/2017 and SAP/203/2017.

The fruits were dried adequately in Sunlight. After complete drying, seeds were removed, and the rest of the fruit was powdered. Triphala powder was prepared from

the fruit wall and pericarp. This powder was stored in a sterilized, dry, airtight container for further study.

3.3 Preparation of plant extract:

For making the plant extract, four different solvents with different polarities were considered. In a 5 litre (L) round bottom flask, 660grams of Triphala powder was soaked in 3L of different solvents (Hexane, Chloroform, Methanol and Water). Then cold extraction of four different solvents in four different flasks was taken. For every 45min, the solution was stirred. After 48hrs, it was filtered, and the extract was collected in a sterilized container. This extract was concentrated by using a Rotary evaporator at 37^0C temperature. In this extract, Hexane, Chloroform, Methanol and Water are in 5.18g, 11.71g, 32.75g and 21.23g respectively. Now, this concentrated extract is stored at 4°C.



Phytochemical analysis flowchart

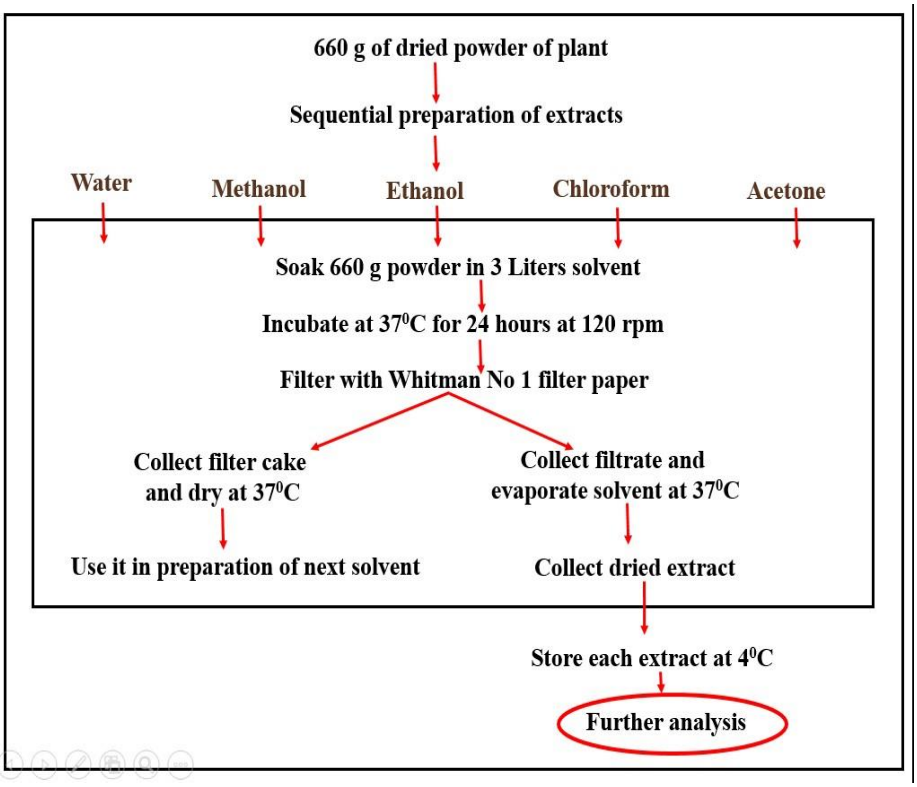


Figure 21. Phytochemical analysis flow chart

3.4 The thin layer of Chromatography (TLC):

With TLC, we can quickly get the polarity of the compounds present in the solution. In TLC, we used a Silica plate having a thickness of 60 F_{254} and a size of 24 * 24 (obtained from Merck). The extract was diluted with Ethanol. TLC plate was marked with the spotting line at 0.5cm from the solution level. In that, 2µl of the sample solution was kept on the spotting line, and the elements were there for 10 min in the moving state. In that, Hexane and Ethyl acetate are in 6:4 ratios, respectively. These two were used in the trial-and-error method.TLC plate was placed in the TLC chamber. After some time, the mobile phase elements started accumulating on the spotting line (to 80%). The plate was taken out from the developing chamber and treated with dry air until the moisture was evaporated from the plate. Under UV light, the spots were observed, which represent different compounds or fractions of the compound. These were developed by anisaldehyde staining method and were preserved for further study.

We can calculate the R_f value using the formula as

R_f = Distance travelled by the solute / Distance travelled by the solvent front with this, we can calculate the R_f value of the compounds and their relative orientation on the plate indicates, the existing polarity between them.

3.5 Gas Chromatography-Mass Spectrometry (GC-MS)

We can easily separate the compounds with the equipment GC-SHIMADZU QP2010. This entire equipment has Elite-1 coated with a Silica capillary coat of 70 eV and Helium gas (colourless&odourless) used as the carrier with 99.99% purity with the continuous flow at 1.51 ml/m was used for measuring at 200°C. 2µl of the sample was taken (split ratio: 20). The oven temperature changed andwas maintained at 70°C (isothermal) for 2 min. The temperature was raised to 300°C for 10 min. The spectra were collected at an interval of 0.5 seconds with a range of 10-1000 m/z. The total run time of GC was 35 min. The percentage of each compound was calculated by comparing the average curve area to the entire area. The percentage was taken with average curve area to total area. The final extract was compared with Retention Time (RT), Retention Indices (RI), mass spectra of WILEY, NIST data (present in its library) present in GC-MS system and Literature data (Nie, Y**,** *et al.,* 2009) and then identified. Based on the database collected from the National Institute Standard and Technology (NIST), Mass-Spectrum and Gas Chromatography was done where it has more than 62,000 patterns. In the NIST library, the known components were already available, and unknown parts were paired. The name of the Test materials, their molecular weight, and the element's structure was appropriately listed.

3.6 Biochemical analysis:

Different people have given different methods in getting the action of phytochemicals (Odebiyi, A, Sofowora, A. E. *et al.,* 1990). Each and every plant has its level of these alkaloids as saponins, steroids, phenolic combinations, glycosides, alkaloids, tannins (Harborne J. B *et al.,*1973). Synthetic tests led to the watery concentrate of each example utilising standard techniques (Edeoga, H.O *et al.,* 2005). The five foci arranged are tried for the nearness of phytochemicals (Kumar, N. S *et al.,* 2017).

3.6.1 Preliminary Phytochemical Screening:

Test for Alkaloids:

3.6.1.1 Dragendorff's Test:

This test is to check the presence of alkaloids in the given solution. In a sterilized beaker 8 gm of $Bi(NO_3)_3$.$5H_2O$ was added to 20 ml of HNO_3 and kept aside. In another sterilized beaker, 2.72 gm of Potassium iodide was added in 50ml of water. These solutions have been combined and allowed to stand still, until the formation of KNO_3 crystals. The supernatant was mixed with water to form a 100 ml solution.

Procedure: An acidic medium was prepared by adding 2ml of HCl to 0.5 ml of Methanolic extract, and Dragondorff's reagent was added. The existence of alkaloids was clear when the residue changes its colour from orange to red.

3.6.1.2 Wagner's Test:

In 5ml of Sulphuric acid, Wagner's Reagent, 1.2 gm of Iodine and 2 gm of Potassium iodide, wasdissolved and diluted with 100ml of distilled water.

Procedure: 1.5% V/v HCl was added to 10 ml of Dragondorff's reagent to make it acidic. To this few drops of Wagner's reagent was added. If it immediately turns to yellow or brown colour precipitate, then it shows the presence of alkaloids in the solution.

3.6.1.3 Mayer's Test:

Mayer's reagent: It involves two different solvents. Solvent one is having 1.36 gm of mercuric chloride & 60 ml of distilled water, and solvent two with 5 gm of potassium iodide & 10 ml of distilled Water. Make it to100 ml by further adding distilled water.

Procedure:

In a test tube, mix dil. HCl (0.2 ml) to Triphala solution (1.2 ml) and add 0.1ml Mayer's reagent. The alkaloid existence was confirmed after seeing precipitate with yellow colour.

3.6.1.4 Flavonoid occurrence test:

Shioda's experiment:In a test, take Triphala solution (0.5ml), dil. HCl (5-10 drops) and ZnCl or Mg (4g), boil it for 2-3 min. When the solution turns to pink, flavonoid presence was confirmed. If the Solution colour turns reddish pink, it denotes that the Triphala extract has flavonoids.

3.6.1.5 Alkaline Reagent Test:

In a test tube, to 1ml of Triphala solution, add dil. Sodium hydroxide, colour changes to yellow. Now titrate with dilute acid till the solution becomes colourless. This shows the presence of flavonoids.

3.6.1.6 Test for Carbohydrates:

For the identification of carbohydrates, the Molisch's reagent was added to 100 mg of Triphala extract in the distilled water (4ml).

Molisch's Test: To the above filtrate, add alcoholic α-naphthol (2-3 drops of 1%) to concentrated Sulphuric acid (2ml) from the sides. A brown ring is formed, which confirms the carbohydrates in the solution

3.6.1.7 Test for Glycosides:

Hydrolyse the Triphala solution for four hours on a water bath to test for Legal's and Bontrager's test to see the glycosides in it.

3.6.1.8 Legal's Test:

In a sterilized test tube, a 4ml amount of hydrolyzed solution was taken. To 3-4 drops of Sodium nitroprusside 1ml Pyridine was declend and mixed well. By adding Sodium hydroxide, the colour changes from pink to red colour. This colour change indicates the presence of glycosides.

3.6.1.9 Bontrager's Test:

In a sterilized test tube, chloroform was added to the hydrolyzed solution and mixed well and remove chloroform as a layer. The glycoside appearance was known by adding the same quantity of ammonia (dilute), which turns pink.

3.6.1.10 Test for Saponins:

1. Add 20 ml of distilled water to the Triphala solution and stir for 15 min till 1cm thick foam is formed. This shows the existence of saponins in it.

2. In another test to show the existence of saponins, a precipitate is formed by adding 1% lead acetate to the Triphala solution (1ml).

3.6.1.11 Test for Tannins:

This will be done in 3 different ways.

- Ferric chloride experiment: To the Triphala solution (2ml), when we add aq. Ferric chloride (5%) the colour changes to violet, which indicates the presence of tannins.

- Experiment for lead acetate: When we add lead acetate (1%) to the Triphala solution (5ml), the colour changes to yellow, which shows that tannins are present in it.

- When we add aq, potassium dichromate (10%) to Triphala solution (1ml), the yellow-brown precipitate is formed showing tannins are present in it.

3.6.1.12 Experiment for phytosterol:

Add alcoholic potassium hydroxide to Triphala solution and dilute it with ether. Once the ether evaporates, the remaining is containing phytosterol.

- Liebermann Burchard Test: The residue was treated with acetic anhydride (3 ml), dilute acetic acid (3-4 drops) and 1 ml of Conc. H_2SO_4. The occurrence of a bluish-green colour ring is an indicative marker of Phytosterols.

- Salkowski experiment. Add Conc. H_2SO_4 (1ml), chloroform (1ml) to the Triphala solution, immediately colour changes to red, showing the presence of phytosterol.

Test for Triterpenoids:

3.6.1.13 Liebermann Burchard Test: The residue was treated with acetic anhydride (3ml), dil. Acetic acid (few drops) and pass Conc.Sulphuric acid through walls. The presence of triterpenoids is confirmed when the solution turns to violet colour.

3.6.1.14 Noller experiment: To the Triphala solution (5mg), add anhydrous stannic chloride (0.01% of 2ml) and pure thionyl chloride. The solution first changes to a purple colour and eventually becomes deep red, indicating triterpenoids.

Experiment for the presence of proteins and amino acids:

3.6.1.15 Ninhydrin Test: Ninhydrin reagent (Triketohydrindene hydrate) was added to Triphala solution, so the colour of the solution changes to purple, which shows the presence of amino acids.

3.6.1.16 Biuret experiment:

Through this experiment, we can easily identify the proteins in the solution. The Triphala solution (1ml) was added to equal volumes of copper sulphate (1%) and NaOH (5%), the colour of the solution changes to purple.

3.6.1.17 Anthraquinones experiment:

The Triphala extract (5ml) was initially hydrolysed with dilute H_2SO_4, and it is extracted with benzene. The addition of 1 ml of dilute NH_3 changes the solution's colour to Rose pink, confirming the existence of Anthraquinones.

3.6.1.18 Test for Phenols

1. Ferric chloride solution (1% aqueous) was added to 5 ml of Triphala extracts. The change of solutions' colour into blue, green, black or purple indicates the presence of Phenols. In certain conditions where an aqueous mixture of Ferric chloride is used, Potassium ferrocyanide has to be added to confirm the test.

3.7 An Antioxidantal Property:

A molecule usually appears when its applications relate to highly reactive oxygen and nitrogen-based biologic functions that radicalise hydroxyl, super oxide anion, peroxide, nitric oxide, peroxynitrite, lipid peroxide, and thiobarbituric acid. The highly reactive radicals oxidatively endogenously convert lipids to lipid peroxyl radicals. Sugar undergoes the process of fermentation to form glyceraldehydes. Nitrogen bases in nucleic acid get modified as hydrogen bonds break, peptide bonds amidst amino acids undergo cleavage, and proteins alter enzymes, and metabolites get changed leading to oxidative stress. Free radicals released by damaging cells (Ray, P *et al.,* 2012) result in releasing free radicals which are of no significance. This occurs during cell metabolism and in response to external factors. If it is difficult for the body to remove the free radicals effectively oxidation stress occurs. This causes harm to cells and their functions. Oxidative stress leads to ageing, respiratory disorders, cataracts, arthritis, brain dysfunctions, immune deficiency, cancer, heart diseases, and other inflammatory conditions. (Lobo V 2010 & Carocho, M *et al.,* 2013)Thus, biological oxidants are formed during various intracellular processes like healthy cellular metabolism,inflammatory reactions, and stimulation of macrophages or by exogenous pollutants or ionising radiations (Pizzino, G *et al.*, 2007). Oxidants accept electrons, a process that causes oxidation at a biological level. Biological oxidation occurs when electrons leave the substance or add at a different atomic or molecular level. The cell destruction caused by unstable free radicals brings the imbalance between oxidants and anti-oxidants. Anti-oxidants are substances that prevent damage caused to the cell by free radicals. As a result, anti-oxidants are also referred to as free-radical scavengers. Endogenous anti-oxidants are those produced by our bodies, whereas exogenous anti-oxidants are those that enter the body from outside sources. It is stressed upon that diet enriched with anti-oxidants is significant to maintain good health. Anti-oxidants external to the body are catechins, beta-carotenes, flavones, flavonoids, lutein, lycopene, manganese, selenium, phytoestrogens, polyphenols, vitamin A, C, E, and zeaxanthin found in the plant-based diet. Natural oxidants save cells from oxidative damage by deactivation and neutralisation of free radicals (Almeida *et al.,* 2011, Gostner *et al.,* 2015).

3.8 Antioxidant studies:

By the presence of secondary metabolites, plants are generally a rich source of antioxidants. If a compound has an unpaired electron in its orbital, which is highly reactive and unstable, is called a free radical, (Ribeiro, J. S *et al.,* 2019). Hydrogen peroxide, Superoxide, Hydroxyl radical, etc. are oxygen radicals. The antioxidant properties of crude Triphala powder wereanalysed by using free radicals DPPH and Hydrogen peroxide. For determining the antioxidant nature of crude Triphala, the Dot blot method was adopted, along with DPPH and Hydrogen peroxide methods.

3.9 Dot blot assay

All four extracts were subjected to dot blot assay to check the antioxidant property (Soler-Rivas *et al.,* 2000). The extracts were diluted using their respective solvents and loaded (5 µl) onto a 5 x 5 cm TLC plate (Silica gel 60 F254, Merck). The plate was air-dried for 3 min and then placed inverted for 10 s in a 0.4 mM DPPH solution. By using the paper, additional solutions are discarded and the plate has been air-dried.Silica plate in TLC chamber was stained, and if it turned to white or pale yellow colour on a purple background, then it indicates the presence of free radical scavengers.

3.10 DPPH method

The free radical scavenging property of four extracts of Triphala was obtained from the DPPH method (Tagashira and Ohtake, *et al.,* 1998). The plant products with different concentrations such as 50, 100, 200 and 500 µg/ml were preserved by mixing with 0.1 mM DPPH in the dark at room temperature for half an hour. We can observe the change in the colour ofthe solution. It was measured at 517 nm using a UV-visible spectrophotometer (Shimadzu 1650pc). This experiment was performed in triplicates by using ascorbic acid as a positive control.

DPPH scavenging activity (%) = ($Abs_{control}$ -Abs_{sample}) x 100/Abs control

3.11 H_2O_2 radical collector:

The H_2O_2radical collecting or removal effect onthe Triphala solution was done by using this method. (Raja Manikandan *et al*., 2011). H_2O_2 at a concentration of 40 mM

was added to a pH 7.4 phosphate buffer. Add crude Triphala extracts in various proportions and incubate them at room temperature for 10 min (50, 100, 250, and 500 g/ml). In the developing mixture, a level of hydrogen peroxide was noticed, which was absent in the phosphate buffer-free growing medium. This was completely controlled by ascorbic acid. This is calculated by using the formula:

Hydrogen peroxide radical scavenging activity (%) = (Abs control - Abs sample) x 100/Abs control

3.12 In silico analysis:

3.12.1 Retrieval of protein structure:

Phospholipase collected from various venoms controls the inflammatory pathway and expansions. For crystallography, Phospholipase from Humans (PDB code: IDB5) is collected from RCSB Protein Data Bank (Berman HM *et al.*, 2002) and taken for X-ray crystallography. Hetero molecules and water were removed and Docking was done for original proteins. Analysis of the protein's Active site was done by using Swiss Protein Viewer (Johansson MU *et al.*, 2012).

3.12.2 Retrieval of ligands:

After GC_MS analysis of Triphala powder, 3D structures of various active compounds were identified and retrieved in SDF format from NCBI Pub Chem Compounds **[National Center for Biotechnology Information**]. Using ChemSpider, 2D structures were sketched. The names and numbers of the compounds are its ID’s (CID:445639) Oleic acid, (544150) Tricyclo, (32801) Androstane, (8141)Nonane, (588380)Methyl 3-ethylpent-2-enoate, (137530) 5-Undecyne, (554144) Methyl 10-methyl undecanoate, (6780) 9,10-Anthraquinone, (5280590) Methyelaidate, (5364768) AC1NSKA3, (5363293) (z)-13-octadecenyl acetate (5281670) Morin (using molinspiration 3D structure was generated). Molinspiraton is cheminformatics software for calculating molecular properties, production of bioactivity, virtual screening, and molecular databases.

3.12.3 Grid preparation and molecular Docking:

Using iGEMDOCK (Hsu, K.C *et al.,* 2011) - Autodock vina, Pyrx and MGL tools, Molecular Docking was performed (Sanner MF *et al.* 1999, Morris GM *et al.,* 2009). In MGL tools, a pop up comes as AutoDock tools. In this, we can create Docking Input files. For performing Docking, selected macromolecules and ligands were chosen. PDBQT files were made by the addition of Kollman charges for protein molecules and by the addition of hydrogen bonds. To give a constant protein structure, a substance was prepared with rotating bonds at PDBQT. For ligands conformations search, a local algorithm named Lamarckian Genetic Algorithm (LGA) was utilized. To generate Grid box protein configuration, files were created by using Cartesian coordinates. The analysis of the docked complex was performed based on hydrogen bonds, the energy present in them (kcal/mol) and the constant that controls (µM) using the Docking tool. All the ligands were ranked based on the docked energy.

3.12.4 Structural and visualization analysis:

Protein and ligand interplay were visualized and analyzed by using the PyMol viewer tool (www.pymol.org) and Discovery studio 2020 (https://www.3dsbiovia.com/).

3.13 Profile of ADME/Tox:

ADME presence is important in an organism for drug metabolism, its absorption, its deposition and removing the excess amount of drugs. With these four steps, ADME maintains the drug performance in the body and its effect on tissues.The toxicological profile is a unique computation of toxicological information on the hazardous substance. They are predicting areas of drug and toxic antagonists at the pre-clinal stage. For the purpose *in silico*, modelling is practised where computer models are developed to model the pharmacological process. This prevents late-stage failure, which saves time and money invested. **(**Durán-Iturbide NA *et al.* 2020**)**

The ADME website (http://www.swisadme.ch/) will help to study the properties of drugs, their structure in the easiest manner. Another site that helps to draw graphs

(http://structure.bioc.cam.ac.uk/pkcsm) for our data gives huge support to the ADME site. A Web interface helps build and manage written content using an easy-to-use visual interface in various modules.

Docking:

As explained in the docking methodology in para 3.12.3 (para no. for the docking process), I took three different chemical drugs to control or cure breast cancer cells. But those have side effects. Anthraquinone and Morin were used (as these are components of Triphala extract) for docking with cancer-causing protein like Human estrogen receptor (2iok) representing the docking results in table 16 (Docking Table).

3.14 Phospholipase A_2 assay in vivo:

With the PLA_2 kit, we can do the PLA_2 assay (Cayman Chemical, Ann Arbor and Michigan, USA). In the reaction, the mixture PLA_2 is present in less quantity as 10µl. We need to add a substrate of 200µl and 10µl of the compound into the well and incubate for 15min.The free thiols combine with DTNB to form 5-thio-2-nitrobenzoic acid. The absorbing capacity at 415nm was given by DTNB (Deng et al., 2013). Thioetheramide PC acts as a positive controlling unit (A.Nataraj, *et al.*2007).

3.15 *In situ* Inhibitory assay of PLA_2 :

Without using the PLA_2 enzyme, PLA_2 activity in breast cancer cell lines was measured with Cayman secretory PLA_2 kit. The maintenance procedure of cell lines is, as mentioned in section 3.14 MDA MB 231 cell lines (Breast Cancer Cell line) were soaked in ethyl acetate (1mg/ml) for 72 hrs.which is the incubation period for the MDA MB 231 cells. After this time, the floating cells have to be collected and measured for the secretory PLA_2. Now, we need to check intracellular PLA_2 by removing and killing the cells. We need to measure and note down the activity of the enzyme for every minute at 414 nm for 15 min through the spectroscope. (Buffer used for killing the cells: 150nM NaCl, 50mM Tris-HCl and 0.1 % Triton X-100).

3.16 Cell culture:

3.16.1 Chemicals:

The chemicals used were in pure form and purchased from various suppliers. Various cancer cells (MCF 10A & MDA MB 231) were taken from The National Centre for Cell Science, Pune, India. The chemicals Poly-L-lysine, 4,6-diamidino-2-phenylindole (DAPI), RNase A, DEAE-cellulose, Propidium iodide, Proteinase K, Phenylmethylsulponyl fluoride (PMSF), Glutaraldehyde, Leupeptin, Aprotinin, Trypsin, Pepstatin A, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lgepal CA-630, EDTA, Sodium bicarbonate, Sodium orthovanadate, Calcium chloride, Hematin, Tween-20, P-actin antibodies, Ponceau S, Triton X-100, IGEPAL CA-630 ordered from Sigma Chemical Company, St.Louis, USA. RPMI 1640, Penicillin, Fetal Bovine Serum (FBS), Phosphate-buffered saline (PBS), Gentamycin and Streptomycin purchased from GIBCO Ltd. (BRL Life Technologies, Inc., Grand Island, NY). Low-fat milk powder ordered from E-Merck. Nitrocellulose membrane ordered from Amersham bioscience corp (Amersham, Bucks, UK). Mouse monoclonal antibodies against Bcl-2, PARP (Poly ADP-ribose polymerase), Bax, Cytochrome C ordered from Santa Cruz, CA, USA. X-ray film and developing solution ordered from Kodak. Acrylamide, Ammonium persulfate, N,N'-Methylenebis-acrylamide, Sodium lauryl sulphate, 5-Mercapto-1-methyltetraxol and Boromophenol blue were ordered from Bio-Rad Laboratories, Richmond, USA.

3.16.2 Cell culture and treatment

The MDA MB 231 cell lines that were not used in the research were preserved in monolayer in Petri dishes in the tissue culture lab. All of the cell lines were cultured in RPMI-1640. Fetal bovine serum (FBS) (10% heat-inactivated), L-glutamine (2 mM), streptomycin (100 pg/ml), and penicillin (100 lU/ml) were added to the media. In a CO2 incubator, these culture plates were kept at 37 °C with 5% CO2.

3.17 Cell multiplication assay:

Cell multiplication (proliferation) was observed by MTT staining (Mossman *et al.,*1983). The MTT assay for viable cells using tetrazolium salt (reduction) & MTT. The dehydrogenases NADH or NADPH as a coenzyme in the mitochondria of cells

reduces the tetrazolium salt in the presence of NADH, or NADPH converts MTT salt (yellow coloured) to formazan crystals (purple coloured) (Liu *et al.,* 1997). After the dissolution of Formazan crystals by organic solvent (DMSO) (Dimethyl sulfoxide) the solution was read spectrophotometrically. The cells were grown in 96 well-plate readers for 24hrs. Each well plate was added with 20µl of MTT (5 mg/ml in PBS) and incubated for four hours at 37°C such that purple-blue coloured precipitate is formed. After dissolving the precipitate in 100 µl DMSO, the MTT number was reduced and was measured by OD at 570nm on Spectrophotometer (p-Quant Biotech instrument, in microtiter plate reader). Each concentration was tested for four replications by three different experiments. After plotting a graph with growth percentage and control, the valuesof mean and standard variations were noted. The Trypan blue exclusion assay counts the viable cells with a haemocytometer.

3.18 Morphological differentiation

MDA MB 231 cells were incubated with 9, 10Anthraquinone (AQ) and Morin. After 24 hrs, the morphological differentiation of cells was observed. The cells were photographed using a Nikon F-601 AF camera and observed using a Phase-Contrast inverted microscope.

3.19 Mitochondrial membrane potential changes ($\Delta\Psi m$) measurement assay

With the use of rhodamine-123 (green Fluorescent Dye) by flow cytometer, membrane potential changes for mitochondria ($\Delta\Psi m$) was calculated. A 24-hour isolated compound penetration (5 µM) was observed for cancer cells (1x106 cells per well). The cells also had rhodamine-123 (200 nM) incorporated and were dark for 35 minutes. These cells were centrifuged and washed with phosphate. This shows the change of the membrane potential of mitochondria (Tong *et al.,* 2004).

3.20 Hoechst 33258 staining of cells for nuclear morphology

Our compounds have been treated with cancer cells (1 X 106 cells/ml/12 well plate) for 24 hours. The cells were washed with saline, centrifugated for 5 min at 300 g, with a phosphate filler pad. In addition, the cancer cells collected from MDA MB 231 have been suspended softly in buffered phosphate (100 µl), fixed with acetic acid and

methanol. Cells grown on the slide were dried at room temperature for 10hrs.Cells that were slipped and kept under subdued light for 30 minutes at room temperature and exposed to the stain of 1 ml Hoechst. The slides were carefully removed to remove excess bleach and fluid (50 μl) mounts (Glycerol, PBS 1:1) and were then covered with a coverslip and sealed with nail polish (before observing under the microscope). Now we can observe morphological changes in the nucleus under a fluorescent microscope (Tong *et al*., 2004).

3.21 Expression of cytochrome c/Bcl-2/Bax through Western blot analysis:

For 24 hours, our compound has been treated with cancer cells. Then cells were washed using PBS and kept in 20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mm of glycerophosphate, 1mM of sodium orthovanadate, 1mM of phenylmethyl fluoride and 10 mg/ml of leupeptin and 20 mg/ml of aprotinin for 30 min at 4 ^{0}Cat 10,000 g for 10 minutes, the buffer was centrifuged. The surfactant extracted contained lysate, used for the cytochrome c calculation. SDS-PAGE (8-12percent) and electrophoresed gel were transferred to the nitrocellulose membrane in an equivalent concentration of the protein from cell lysate. In order to avoid unspecific binders, the diluted antibody buffered with Tris-buffered saline was blocked by a membrane (5%) with the aim of preventing the incubation with unspecified bindings of 4^{0}C for 8-12 hours (0.05% to 20% twice and 5% fat-free dry milks). Cytochrome C/Bcl-2/Bax blocked the primary antibodies. Through the western blotting technique, we can identify the cell growth with secondary antibodies. (Reddy *et al*., 2009).

3.22Caspases assay:

In accordance with manufacturer instructions, caspases-3, -8 and -9 were checked using a caspase-colourimetric protease package (Abcam, Cambridge, MA, USA). The cells were covered with the substance for 48hrs and by adding 50°C chilled lysis buffer, cells were lysed and grown on ice (10min). Centric lysate was produced at 10.000 g for 1 minute, and the supernatant was extracted. Protein-contaminated cell lysate (DEVD-pNA), IETD-pNA, LEHD-pNA, and 4 ml 4 mmol/L pnA combined substrate wereincubated at 37°C during 3hours, respectively (caspases 3,-8 and -9).A 405 nm (Bio-rad) ELISA microplate reader has calculated the released pNA amount.

Caspase operation was described as a folding distinction (Aysegul *et al.*,2012 and Heydar *et al*., 2013).

3.23 DNA fragmentation assay:

The vehicle alone (AQ and Morin) has incubated the cells for 24 hours. DNA leaf training takes place. The SDS or Proteinase K, or RNase were used to extract isolated DNA fragments. The isolation of pure, genomically contaminated DNA occurs (Hermann *et al.,* 1994). The cells (5 x 10^6) were pelleted, washed in cold PBS with a buffer of EDTA (1 mM), Tris-HCI (pH 8.0) and Triton X-100 (0.2%) for 20 minutes at 4°C. The pellets were fed into the buffer. Centrifugation of this solution at a speed of 14000 x g took place for 15 min. Proteinase K (0.5 pg/ml) and 1 percent SDS (sodium dodecyl sulfate) were treated for 1 hour at 50°C for supernatant. Two times the ethanol precipitate was collected and used to get DNA with 140 MCI at -20°c for complete night. It was washed with 70% ethanol and dissolved into TE with RNase A for an hour at 37°C. 15 µl DNA was piped with the sample buffer DNA and mixed with 3µl (30 percent glycerol, 0.25 percent xylene cyanol and 0.25 percent bromophenol blue). Then we get a 1% agarose gel in TBE buffer with a 100 bp DNA strand (44.6 mM Tris, 1 mM EDTA and 44.5 mM boric acid). DNA fragments were manufactured using Ethidium bromide (0.05 mg/ml) and observed under UV light. Apoptosis is concluded by the division of the DNA with the oligonucleosomal ladder (multiple of about 180-200 bp).

3.24 Quantification of Apoptosis by Flow Cytometry Apoptosis

Apoptosis was measured by Propidium iodide, which allows the contents of nuclear DNA by flow cytometer to be quantified (Reddy, M. C *et al.,* 2003). The DNA histograms sub-G1 events are critical for the analysis of apoptotic activity (Nicoletti *et al.,*1991). G1 cells, which can be seen with an inflow of cytometric histograms (G0/G1 sub-cells) are more stained than apoptotic cells. MDA MB 231 (3.5 X106 cells) were plated in six cultivated plates and added with 10 percent FBS with or without AQ and Morin for 24 hours, respectively.Following this treatment, a cell suspension of 200 µl PBS, 2 ml ice-cold ethanol (70% was used for fixation) was prepared and kept at 4°C overnight. The cells were centrifuged with 500xg for 10min,

the floating part was removed. Now the pellets are treated with RNase A (100 pg/ml) and Triton X-100 (0,1%) in a 500 µl PBS supplement and incubated at 37 °C for 30 minutes. Moreover, PI (50 pg/mL) was stained and darkly incubated at 4°C for 30 minutes.A FACS Calibur flow cytometer measured the red fluorescent light at 530 nm (at excitation) and 615 nm (emission) of individual cells (Becton Dickinson, San Jose, CA, USA). A minimum of ten thousand case studies has been taken for each sample. The trypan blue exclusion method was followed in order to analyse viability.

3.25 Statistical Analysis

Statistical analysing of data was done using the mean and SEM (Standard Error of the Mean) (n=3). The significant difference (P) obtained with unpaired students t-test. $P \leq 0.05$ is the standard value for a significant difference.

CHAPTER - 4
RESULTS AND DISCUSSION

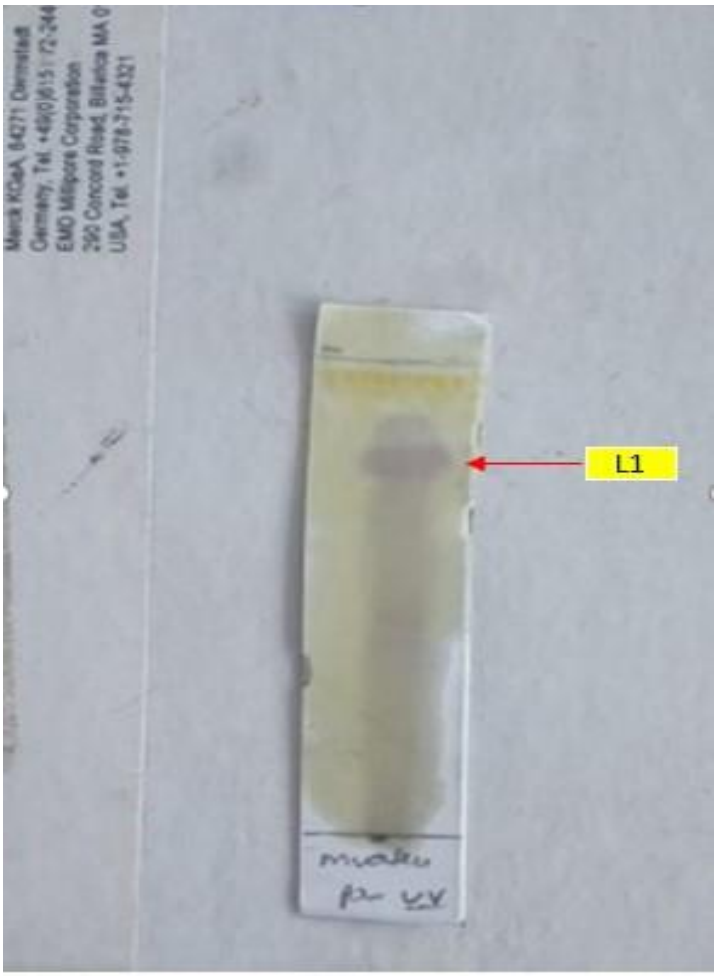
4. Plant materials:

Fruits of *Phyllanthus emblica, Terminalia chebulaand, Terminalia bellirica* are collected from the Vikarabad forest (Dist.Vikarabad), Telangana State. The fruits are kept for drying. After complete drying of the fruits, seeds are removed. The dried pericarp is made as powder and preserved in a sterilized, airtight container. It is called Triphalachuran or powder. Triphala powder was used for further experiments. These plants were recognized and identified by BSI of Deccan Regional Centre, Hyderabad **(No. BSI/DRC/17-18/TECH/272).** These specimens are preserved at the Department of Botany, SAP Degree & PG College, Vikarabad (Dist.Vikarabad), Telangana State. The voucher numbers are SAP/201/2017, SAP/202/2017 and SAP/203/2017.

4.1 Thin Layer of Chromatography (TLC):

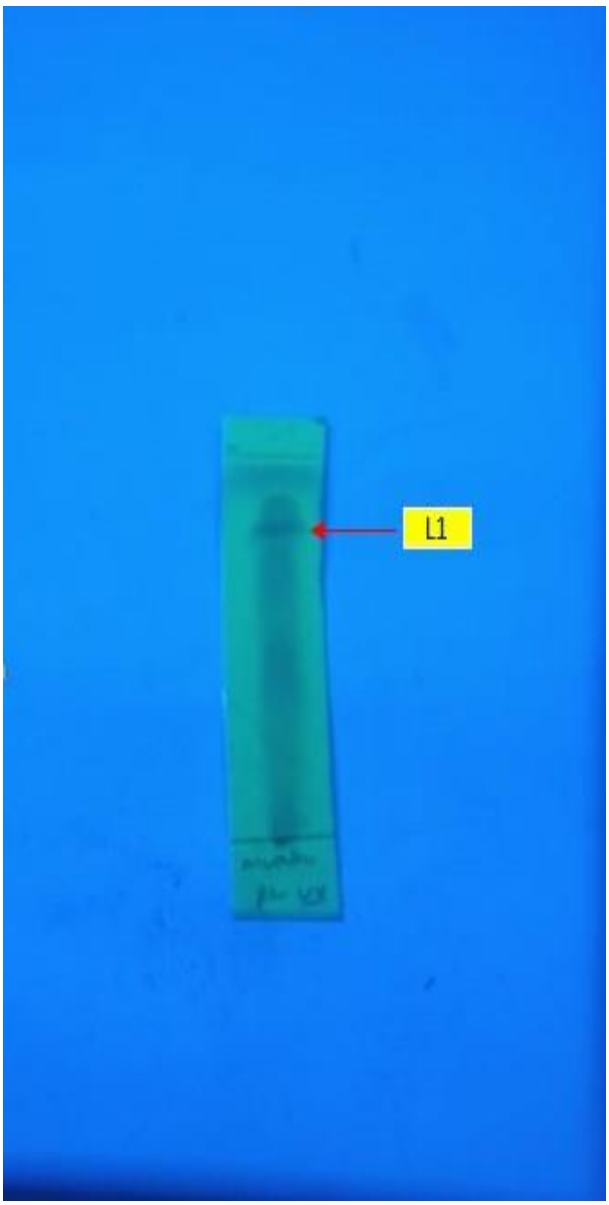
One of the methods of chromatography for plant extract is TLC (Banu and Nagarajan, 2014). For studying the mixture of the compound in plants, TLC is simple, quick and less expensive. TLC method needs two different types of particles as stationary and mobile phase particles. In the stationary phase, divided particles are present, and the mobile phase is a mixture of solvents. Cellulose powder, polyamides, silica gel having chemical bonds, alumina, plastic, ion exchangers or metallic plates are examples of stationary phase. Here, we are using TLC silica sheet and ethyl or hexane acetate as stationary and mobile phase particles, respectively. For the identification of polarity and the number of compounds present in four different extracts, we use TLC made from mice. Place this sample on the silica plate, which is kept in a mobile phase solution. Different solvent ratios are made with various solvents, and ethyl or hexane acetate splits and produces compounds from all extracts. With varying ratios of ethyl or hexane acetate from 10:0 to 1:9 (10 ml mobile phase), the mobile phase polarity can change. The compounds can be separated easily at a 6:4 ratio of ethyl or hexane acetate. These compounds were stained with anisaldehyde and observed at UV 254 nm.

The Triphala fruit extract was separated for its compound using four different solvents like hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate ($C_4H_8O_2$), ethyl alcohol (C_2H_5OH) which were capable of dissolving and dispersing one or more substances. Compounds extracted using hexane and ethyl alcohol as a solvent had lesser concentration rather than the compounds extracted when used dichloromethane and ethyl acetate. The compound separated from the extract using the solvents dichloromethane and ethyl acetate had Rf value 0.8, 0.7,0.5,0.3,0.2. The Rf value gave a complete insight into the character of the compound and is used to identify the compound as Distance travelled by the compound to the distance travelled by the solvent.



(With normal light-occurrence of TLC plate)

Fig. 22 Partial characterization of Phyto constitution from Triphala Powder extract by TLC

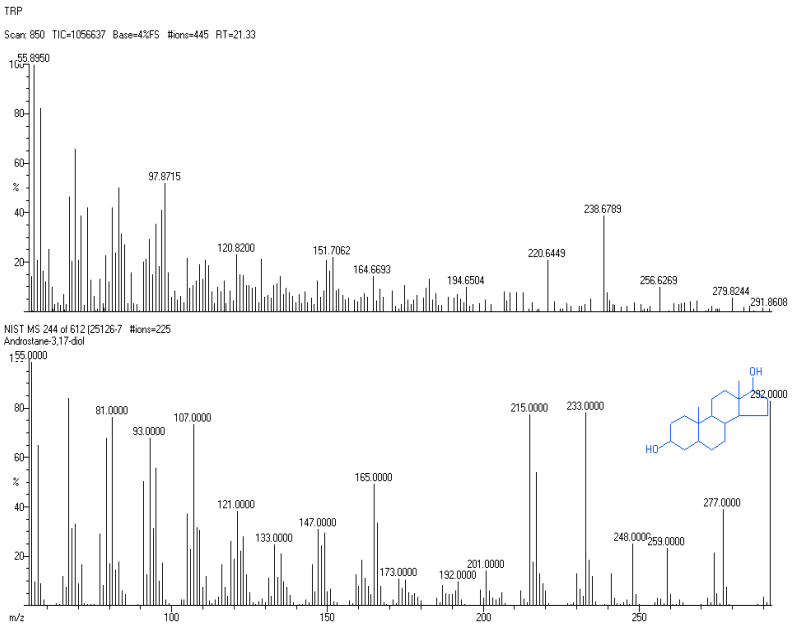
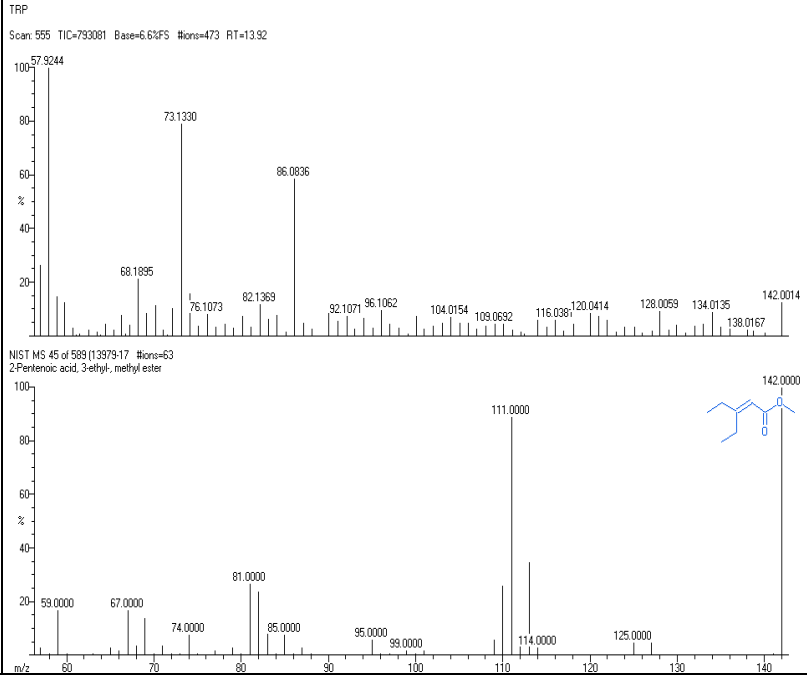


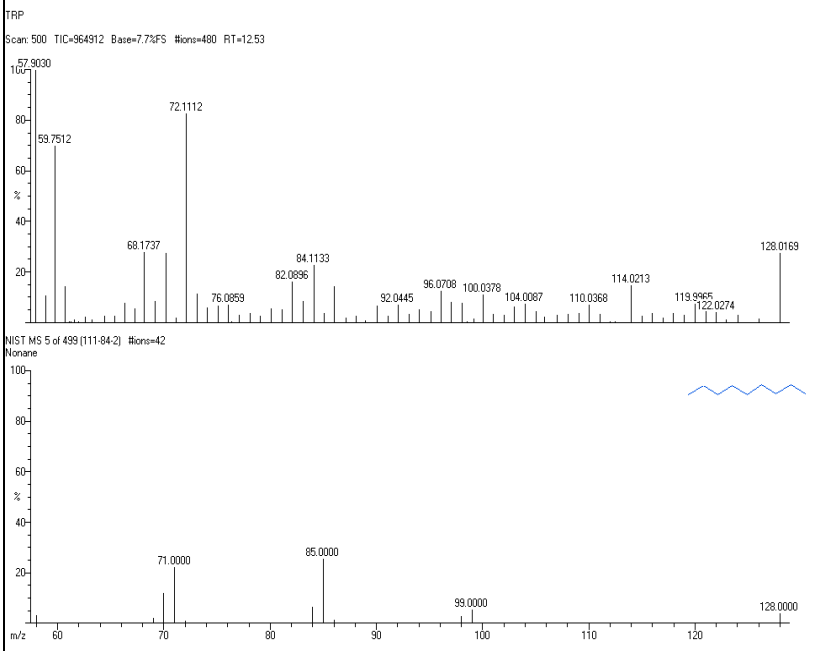
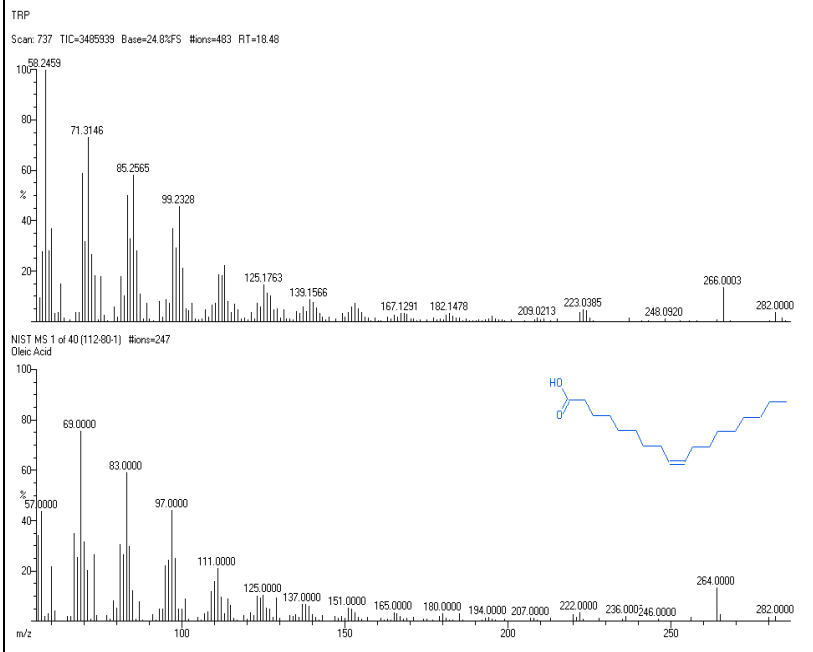
With UV-light (360nm)-occurrence of TLC plate

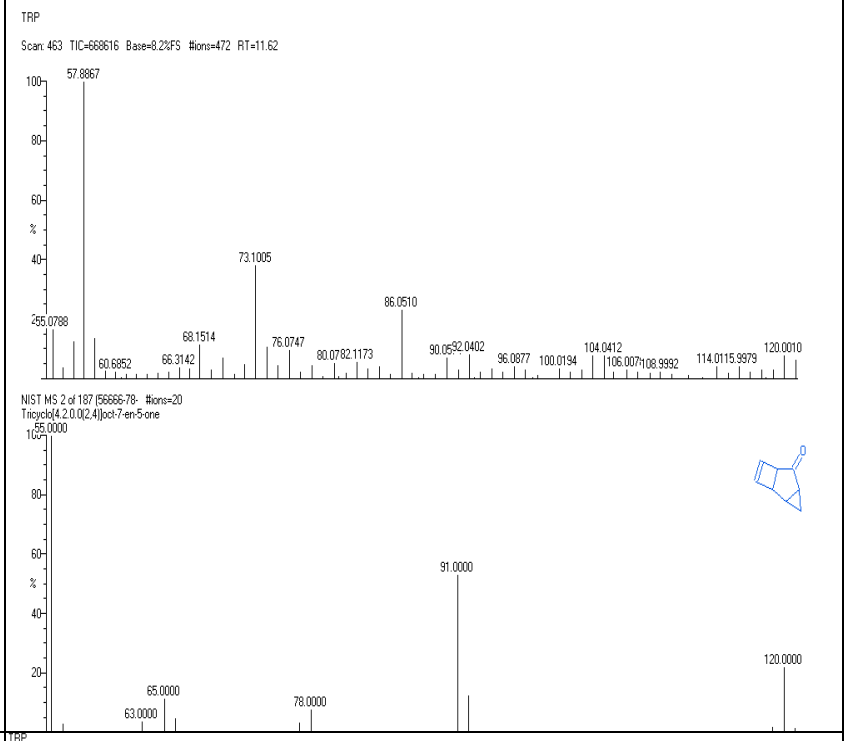
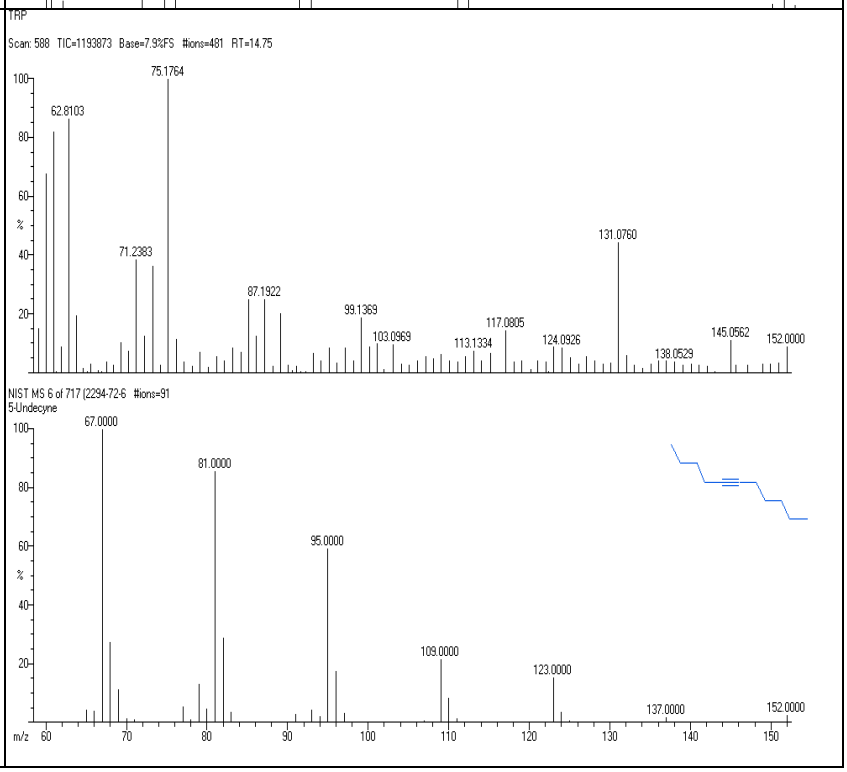
Fig. 23 Partial characterization of Phyto constitution from Triphala Powder extract by TLC

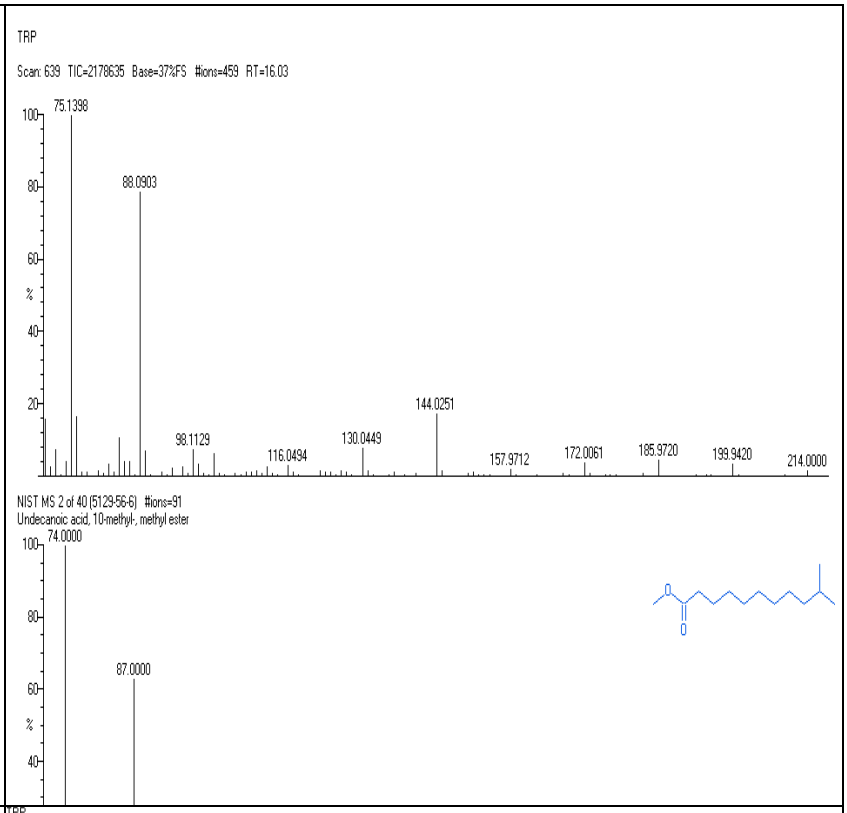
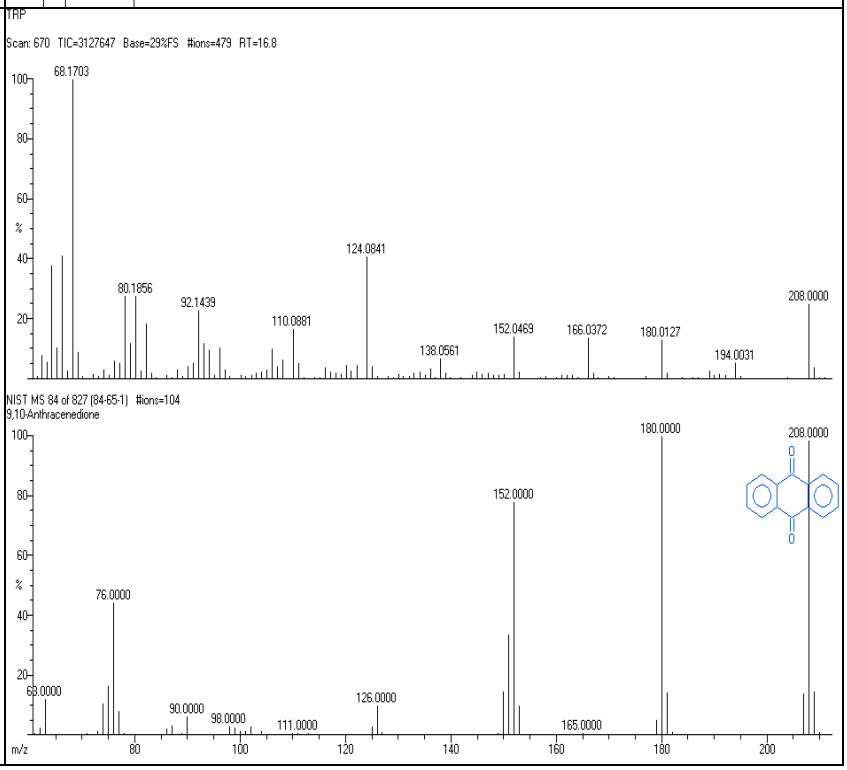
4.2 Gas Chromatography to isolate Triphala extract compounds

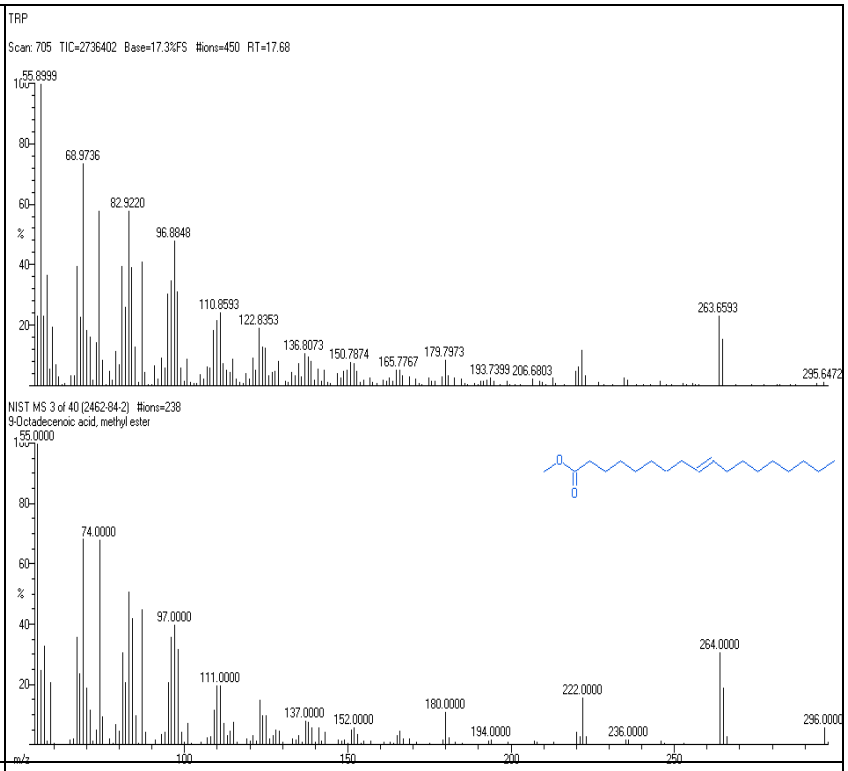
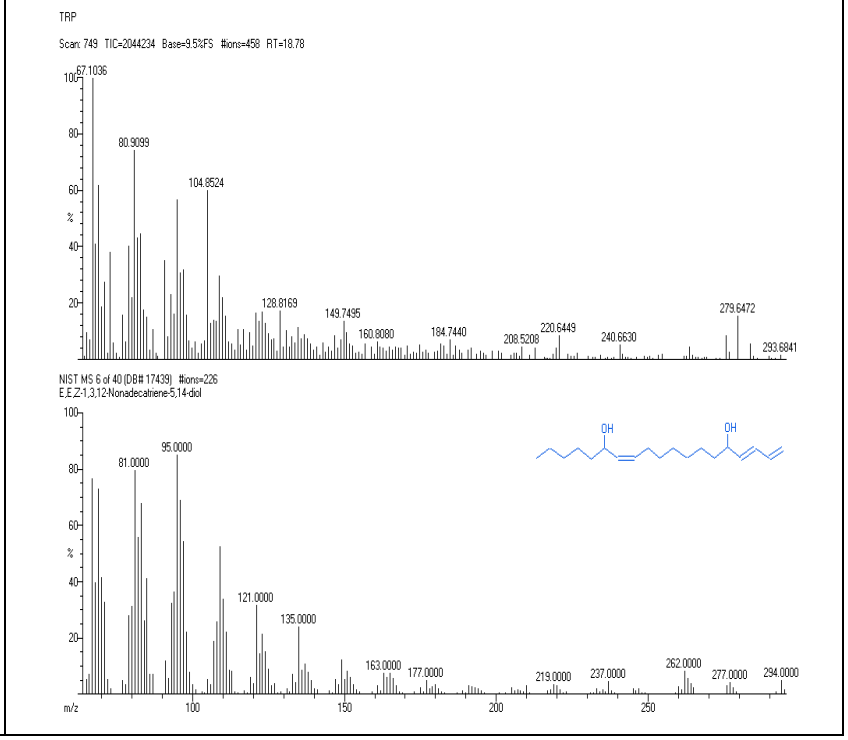
Using the gas chromatography technique, it is analysed that Triphala extract comprises of 12 different compounds which were tested for their purity and thus separated as a part of analytical chemistry.

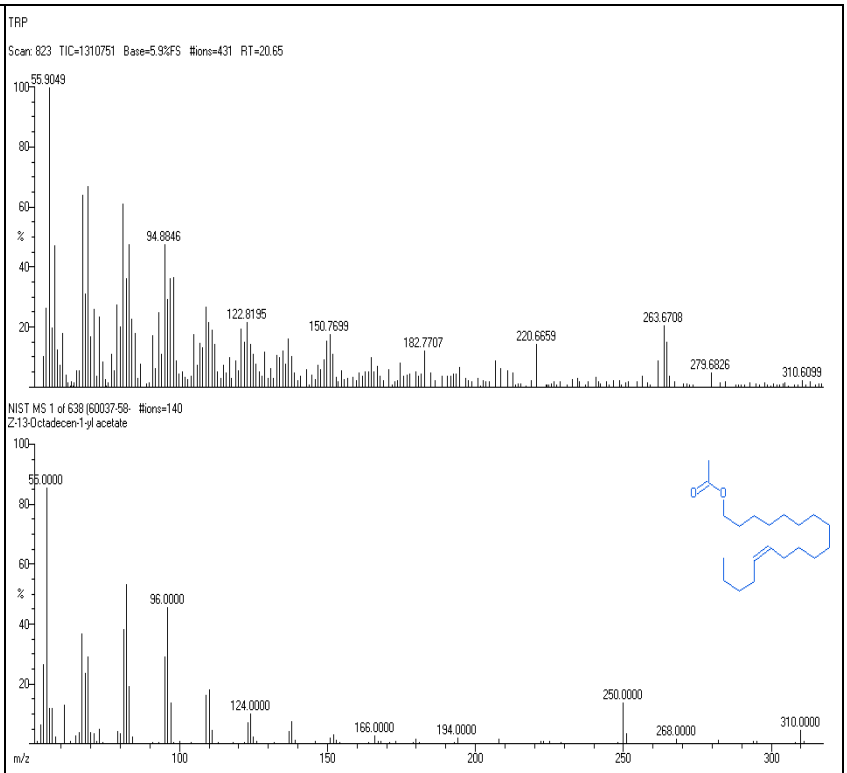
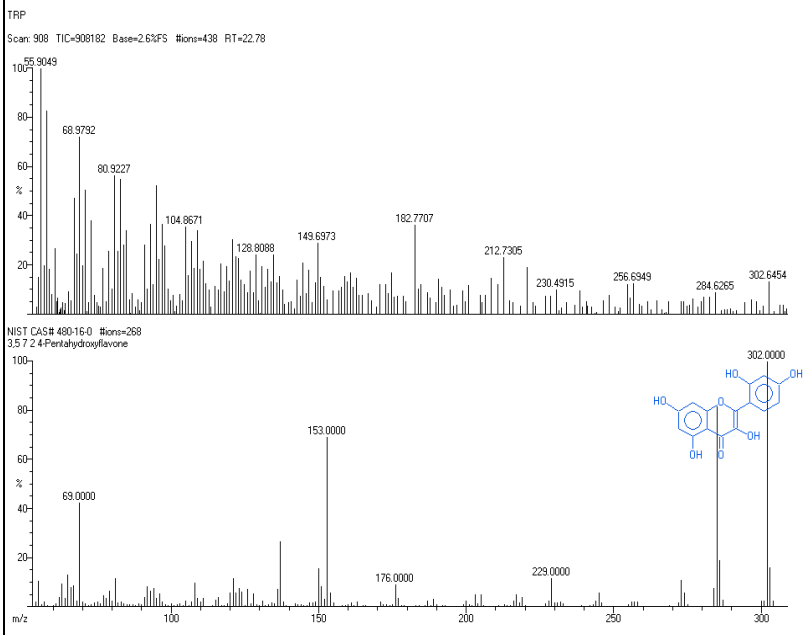
GC_MS Code **25126-7** **PubChem CID code** **32801** **IUPAC Name** **1. Androstanediol**	
GC_MS Code **13979-17** **PubChem CID code** **588380** **IUPAC Name** **2. Methyl 3-ethylpent-2-enoate**	

GC_MS Code **111-84-2** **PubChem CID code** **8141** **IUPAC Name** **3. Nonane**	
GC_MS Code **112-80-1** **PubChem CID code** **445639** **IUPAC Name** **4. Oleic acid**	

GC_MS Code **56666-78** **PubChem CID code** **544150** **IUPAC Name** **5. Tricyclo [4.2.0.02,4] oct-7-en-5-one**	
GC_MS Code **2294-72-6** **PubChem CID code** **137530** **IUPAC Name** **6. 5-Undecyne**	

GC_MS Code **5129-56-6** **PubChem CID code** **554144** **IUPAC Name** **7. Methyl 10-methylundecanoate**	
GC_MS Code **84-65-1** **PubChem CID code** **6780** **IUPAC Name** **8. 9,10-anthraquinone**	

GC_MS Code **2462-84-2** **PubChem CID code** **5280590** **IUPAC Name** **9. Methyelaidate**	
GC_MS Code **DB#17439** **PubChem CID code** **5364768** **IUPAC Name** **10. E,E,Z-1,3,12-Nonadecatriene-5,14-diol**	

GC_MS Code **60037-58** **PubChem CID code** **5363293** **IUPAC Name** **11. (z)-13-octadecenyl acetate**	
GC_MS Code **480-16-0** **PubChem CID code** **5281670** **IUPAC Name** **12. Morin**	

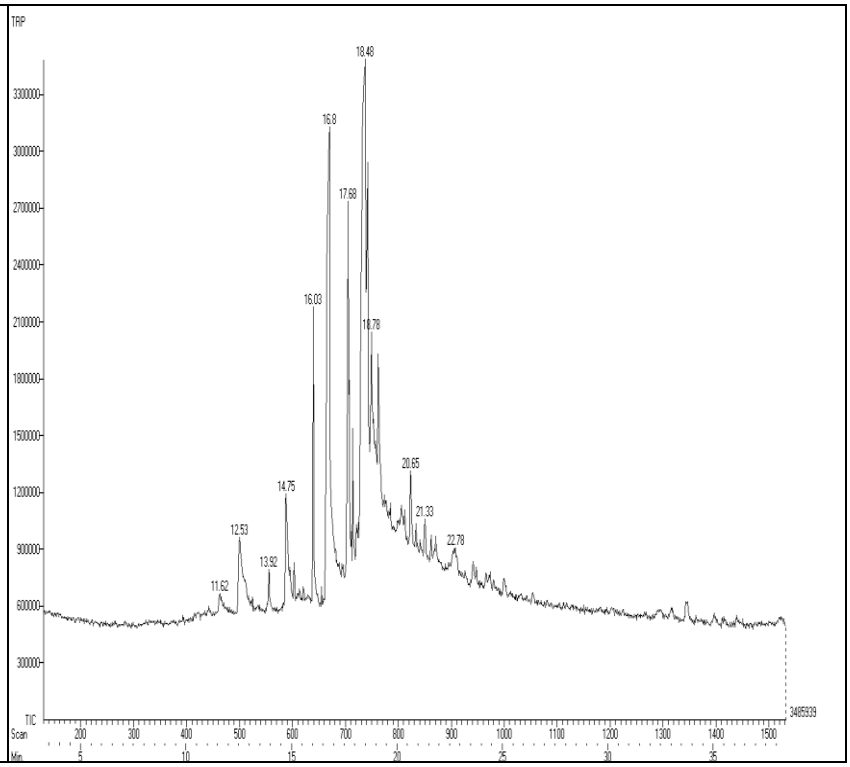
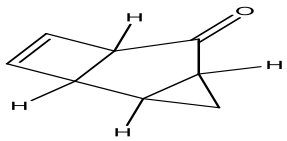
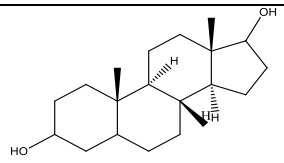
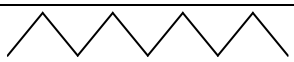
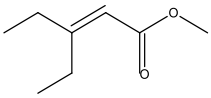
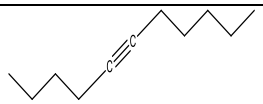
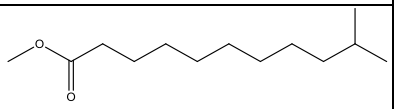
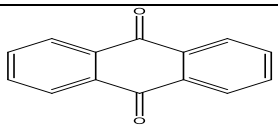
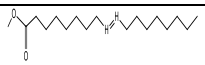
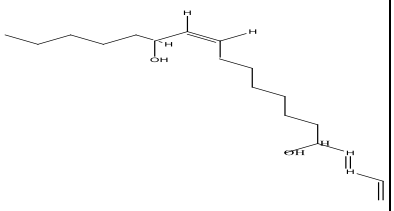
Triphala powder analysed by Gas chromatography-mass spectrometry (GC_MS)	

Fig. 24 Characterisation of Triphala compound analysed by GC_MS.

Table.9 Different Molecular structures and theirPubChem codes isolated from Triphala powder from GC_MS

S. No.	GC_MS Code	PubChem CID code	IUPAC Name	Chemical formula
1.	112-80-1	445639	Oleic acid (abundant fatty acid)	
2.	56666-78	544150	Tricyclo[4.2.0.02, 4] oct-7-en-5-one	

3.	25126-7	32801	Androstanediol	
4.	111-84-2	8141	Nonane	
5.	13979-17	588380	Methyl 3-ethylpent-2-enoate	
6.	2294-72-6	137530	5-Undecyne	
7.	5129-56-6	554144	Methyl 10-methylunde-canoate	
8.	84-65-1	6780	ANTHRAQUINONE	
9.	2462-84-2	5280590	Methyl elaidate	
10.	DB#17439	5364768	AC1NSKA3 or E,E,Z-1,3,12-Nonadecatriene-5,14-diol	

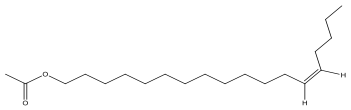
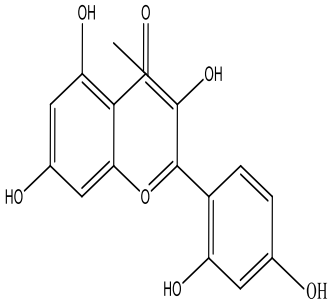
11.	60037-58	5363293	(z)-13-octadecenyl acetate (or) 13-Octadecenyl acetate, (13Z)-	
12.	480-16-0	5281670	Morin	

Table. 10 Different Molecular name and molecular weight and its Pubchemcodes isolated from Triphala powder from GC_MS

S.No.	GC_MS Code	PubChem CID code	IUPAC Name	Chemical formula	Mol.Wt.
1.	112-80-1	445639	Oleic acid	C18H34O2	282.468 g/mol
2.	56666-78	544150	Tricyclo	C8H8O	120.151 g/mol
3.	25126-7	32801	Androstane	C19H32O2	292.463 g/mol
4.	111-84-2	8141	Nonane	H3C-(CH2)7-CH3 or C9H20	128.259 g/mol
5.	13979-17	588380	Methyl 3-ethylpent-2-enoate	C8H14O2	142.198 g/mol
6.	2294-72-6	137530	5-Undecyne	C11H20	152.281 g/mol
7.	5129-56-6	554144	Methyl 10- methyl undecanoate; Methyl isolaurate;	C13H26O2	214.349 g/mol

8.	84-65-1	6780	ANTHRAQUINONE	C6H4(CO)2C6H4 or C14H8O2	208.216 g/mol
9.	2462-84-2	5280590	Methyelelaidate	C19H36O2	296.495 g/mol
10.	DB#17439	5364768	AC1NSKA3	C19H34O2	294.479 g/mol
11.	60037-58	5363293	(z)-13-octadecenyl acetate	C20H38O2	310.522 g/mol
12.	480-16-0	5281670	Morin	C15H10O7	302.238 g/mol

1. Oleic acid is an odourless, colourless, naturally occurring fatty acid having an empirical formula of C18H34O2 and a molar mass of 282.468 g/mol. It has a CID code No.445639 in the world's largest free chemistry database called PubChem. Its GC_MS code is 112-80-1.
2. Tricyclo [4.2.0.02,4]oct-7-en-5-one with a PubChem CID 544150 has a molecular weight of 120.15 g/mol. Its molecular formula is C_8H_8O. Tricyclo GC_MS code is 56666-78.
3. Androstane-3,17-diol with PubChem CUD 32801 and has a molecular formula of $C_{19}H_{32}O_2$ is a 17 hydroxysteroid, a three hydroxysteroid and an androstanoid. It is a metabolite of testosterone with androgenic activity pertaining to the development of male characteristics. Its molecular weight is 292.463 g/mol, and GC_MS code is 25126-7
4. Nonane has a PubChem CID 8141 with a molecular formula of C_9H_{20} having a molecular weight of 128.25g/mol nonane is a linear alkane hydrocarbon which is a colourless flammable liquid.
5. Methyl 3-ethylpent-2-enoate with a PubChem CID 588380 having a molecular formula $C_8H_{14}O_2$. Its molecular weight is 142.2g/mol. Its GC_MS code is 13979-17
6. 5-Undecyne with a PubChem CID 137530 and a molecular formula $C_{11}H_{20}$. Its molecular weight is 152.28 g/mol. Its GC_MS code is 2294-72-6

7. Methyl 10-methyl undecanoate its PubChem CID code 554144 and its GC_MS code is 5129-96-6 its chemical formula is $C_{13}H_{26}O_2$ and molecular weight is 214.349 g/mol
8. Anthraquinone is an aromatic organic compound with a formula of $C_{14}H_8O_2$ with active components of plant blends used as medicines. It PubChem CID code 6780 and GC_MS code is 8465-1. It has a molecular weight of 208.216 g/mol.
9. Methyl elaidate has a PubChem CID code 5280590, and its GC_MS code is 2462-84-2; its empirical formula is $C_{19}H_{36}O_2$ and molecular mass of 296.495 g/mol.
10. E, E, Z-1,3,12-Nonadecatriene-5,14-diol has a empirical formula of $C_{19}H_{34}O_2$. Its molecular weight is 294.479 g/mol. It is PubChem CID 5364768, and GC_MS code is 17439.
11. (z)-13-Octadecenyl acetate has a PubChem CID code 5363293, and the GC_MS code is 60037-58. Its chemical formula is $C_{20}H_{38}O_2$ and having a molecular weight of 310.522 g/mol.
12. Yellow coloured Morin was extracted from the plant body. It is a Pentahydroxyflavonewith 7 hydroxy flavanols having three oral hydroxyl groups at positions 2,4 and 5 act as anti-oxidant, a metabolite, an anti-hypertensive, a hepatoprotective agent, a neuroprotective agent, an anti-inflammatory agent, an antineoplastic agent and an angiogenesis modulating agent. It has a chimeric formula of $C_{15}H_{10}O_7$ and a molecular mass of 302.238 g/mol. Its GC_MS code is 480-16-0, and the PubChem CID code is 5281670.

4.3 Phytochemical analysis:

Phytochemicals protect the organism from chronic diseases. Triphala powder is used with five different solvents like water, chloroform, acetone, ethanol and fuel for knowing the list of phytochemicals in it. Initially, the phytochemical analysis was observed with various elements. The elements are tannins, carbohydrates, terpenoids, steroids, flavonoids, alkaloids, oils, saponins, internal organ glycosides, coumarins, mucilage and gum.

Table 11: Results of the phytochemical evaluation of Triphala plant extracts

Tests Conducted	Solvents Used				
	Water	Acetone	Chloroform	Methanol	Ethanol
	Identification of Carbohydrates				
Molisch's Test	+	-	-	-	-
	Test for Tannins				
Ferric chloride Test	+	+	-	+	+
	Test for steroids				
GC_MS test	-	+	+	+	+
	Test for Terpenoids				
Salkowski Test	+	+	+	+	+
	Identification of alkaloids				
Mayer's Test	+	-	+	+	+
	Identification of Flavonoids				
Alkaline Reagent Test	+	+	-	-	+
	Identification of Proteins				
Xanthoprotein Test	-	+	+	-	+
	Identification of cardiac Glycosides				
Keller Killani Test	-	+	-	-	-
	Test for fixed oils				
Translucent Test	-	-	-	-	-

	Test for saponins				
Foam Test	+	+	-	-	-
	Test for Phenolic Compounds				
$Fecl_3$ Test	-	+	-	+	+
	Identification of Coumarins				
Phytocatalysis	+	+	+	+	+
	Test for Amino Acid				
Ninhydrin Test	-	-	-	-	-
	Test for gums and mucilage				
O- Toluidine Test	-	-	-	-	-

Five different extracts phytochemical study gives some other results for tannins, carbohydrates, internal organ glycosides, steroids, flavonoids, coumarins, synthetic resin compounds and alkaloids. There is a great importance of artificially made resin compounds. They help cellular materials to create similar phenol complex compounds. The phenol complexes participate in making plasma membrane (semi-permeable membrane) which associate with Nursing (Gupta VK, &Singh GD *et al.*,2010). Various biologically active components protect the organism from the stress occurred by using oxygen. Sometimes, it may cause multiple infections such as ageing, problems related to causes cancer (Robards K, *et al.*,1999).

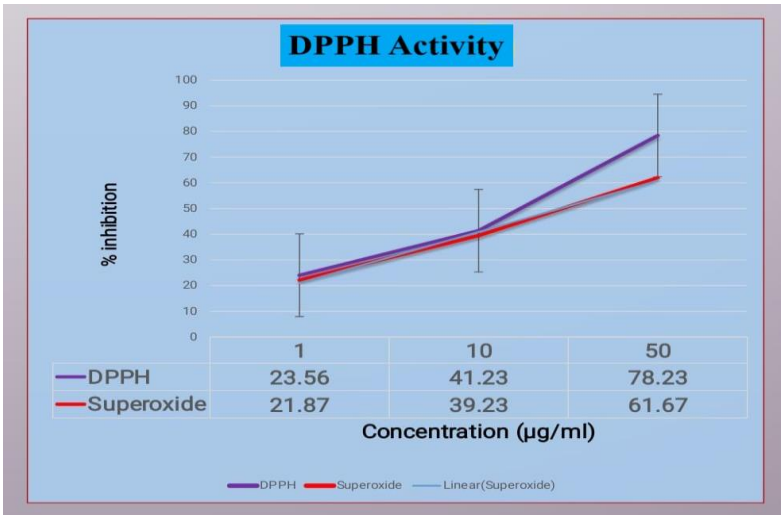
Usually, saponins are used in making pesticides, detergents, controlling molluscs, used in industries (for foam making, surface-active agents etc.,) and used in curing some health problems (Arunasalam JK *et al.,* 2004). Flavonoids guard the cells by controlling the cell signalling pathway. Tannins are used as a growth regulator, pesticides and naturally protect plants from grazing animals.

4.4 Radical scavenging activity of DPPH:

The extract of methanol mixed Triphala powder has different radical removing action of DPPH. Controlling percentage is 23.5, 39.23, 78.25 at 1, 10, 50µg/ml respectively. The IC 50 value of seed coat extract is 23.12µg/ml shows varying from the standard superoxide values of 32.66µg/ml.

Table 12 Results of Radical scavenging activity of DPPH

Sample	Concentration of extract (µg/ml)	Percent of inhibition	IC50 (µg/ml)
DPPH radical scavenging activity			
Triphala powder	**1µg/ml**	**23.50**	**23.12**
	10µg/ml	**41.23**	
	50µg/ml	**78.23**	
Superoxide	**1µg/ml**	**21.87**	**32.66**
	10µg/ml	**39.23**	
	50µg/ml	**61.67**	



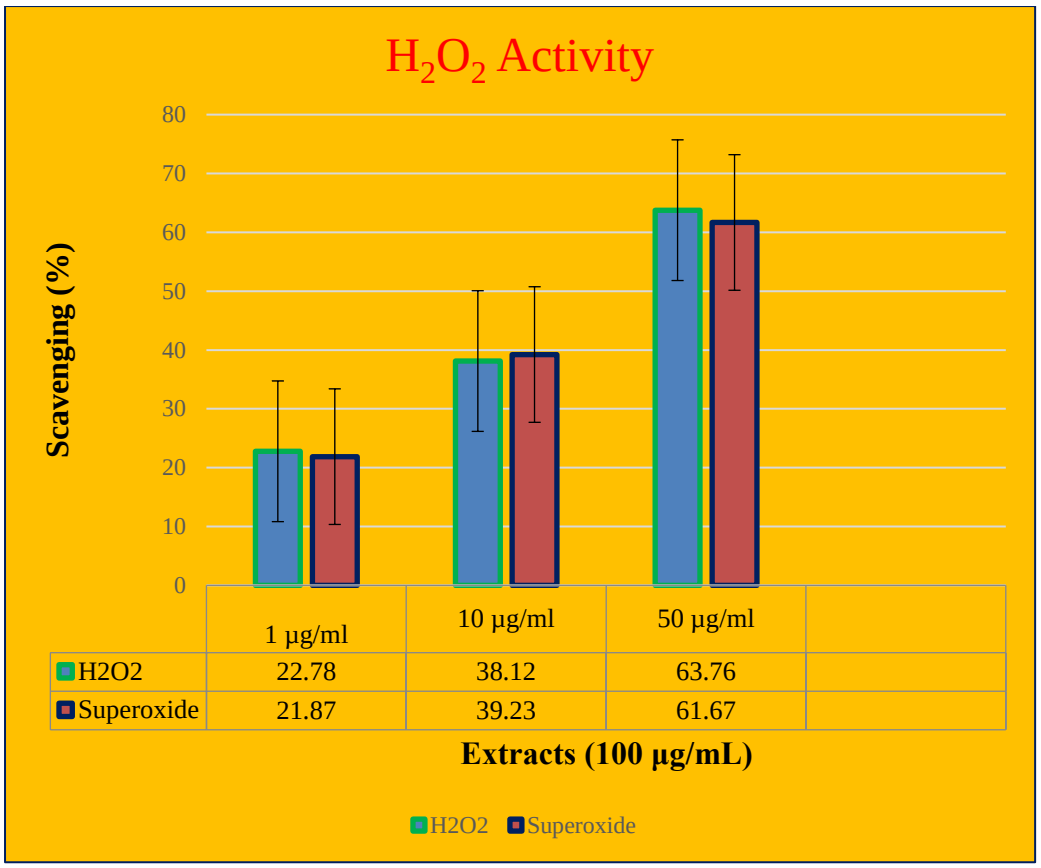
Graph 1. Shows the DPPH activity of Triphala powder extract

4.5 Hydrogen peroxide scavenging assay:

Polyphenols are more in Triphala powder. They act as natural scavengers. Along with IC 50, hydrogen peroxide was added with a concentration of r2 = 0.9581 to Triphala powder (**Table 13**). The value of IC 50 for hydrogen peroxide is 31.18 µg/ml and for ascorbic acid is 32.66 µg/ml (r2 = 0.9254, Fig. 6). Hydrogen peroxide immobilizes few enzymes like the thiol group by oxidation. It is a weak oxidizing agent.

Table 13 Results of Hydrogen peroxide scavenging assay

Sample	Concentration of extract (µg/ml)	Percent of inhibition	IC50 (µg/ml)
DPPH radical scavenging activity			
Triphala powder	**1µg/ml**	**22.78**	**31.18**
	10µg/ml	**38.12**	
	50µg/ml	**63.76**	
Superoxide	**1µg/ml**	**21.87**	**32.66**
	10µg/ml	**39.23**	
	50µg/ml	**61.67**	



Graph 2. Shows the H_2O_2 activity of Triphala powder extract

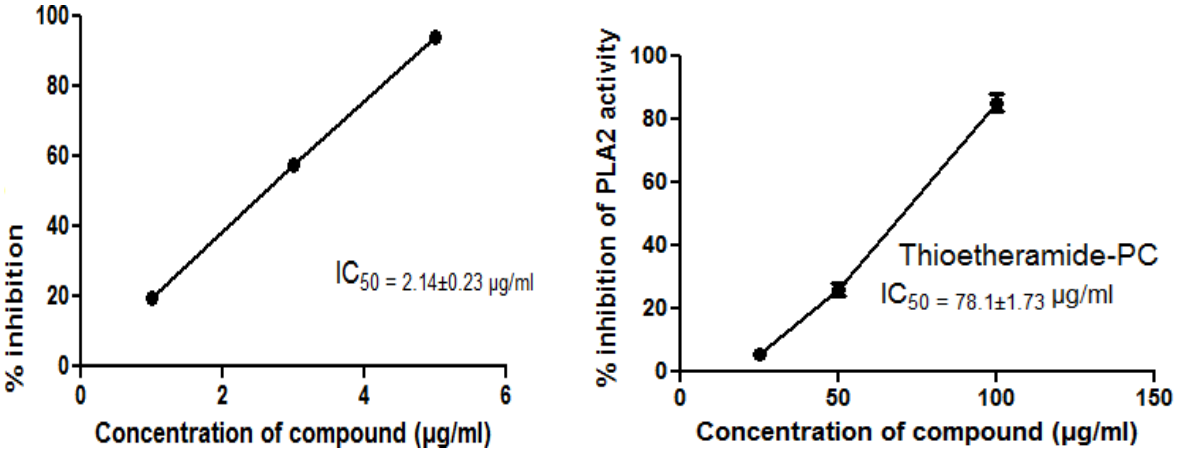
It can quickly move among the membranes and through intercellular spaces. It forms hydroxyl radicals after the reaction with Fe^{+2} and Cu^{+2} cations. Hydroxyl radicals have the huge oxidizing capacity, but with phenolic groups, it acts negatively. So, it releases hydrogen atoms and acts as a free radical scavenger.

4.6 Results of PLA_2 assay:

The controlling strength of Triphala powder on PLA_2 acts with different concentrations in ethanol in µg/ml observed in vitro conditions. These results are compared with Thioethramide–PC, which controls more efficiently PLA_2 movement (Table 14). The digitally calculated IC50 values are on a Microsoft Excel sheet. The IC50 value for positively acting Thioethramide – PC is 78.1 µg/ml and for Triphala powder is 2.14 µg/ml. We can thus conclude that Triphala powder can control phospholipase A_2 activity and can be used for treating inflammations and oncological problems.

Table 14 Effect on Triphala plant extract on PLA_2 assay

S.NO	NAME OF THE PLANT	PLANT PART	IC50 (µg/ml)
			PLA_2 enzyme
1.	Triphala is the combination of three plants (*Phyllanthus emblica, Terminalia chebula, Terminalia bellirica*)	Fruit wall, Pericarp	2.14
2.	Positive control Thioetheramide-PC		78.1



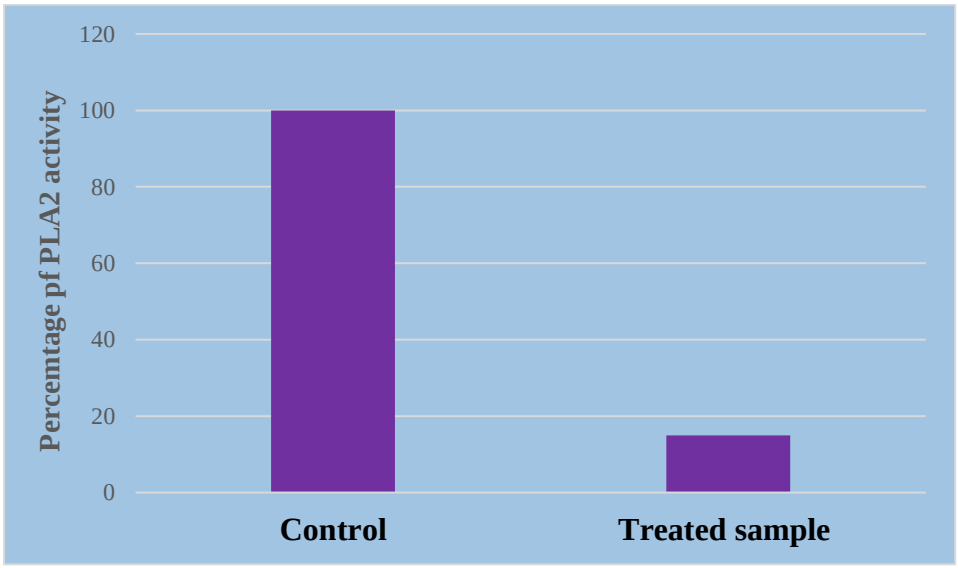
Graph 3. Effect of Triphala on PLA_2 activity

4.7 *In situ* inhibitory nature of PLA_2:

From all the Triphala powder abstracts, ethyl acetate abstract has an excellent capacity to control PLA_2 enzyme activity. So, it was further tested spectroscopically on PLA_2 present in the breast cancer cell lines by using the Cayman Kit.

The evaluation was done by the sample, which does not have plant extract (only cells are present) that controls enzyme activity. Still, the example used for treatment has an

expectation of 1 µg/ml of ethyl acetate (ethyl acetate + cell lines extract). Leave it undisturbed for 72 hrs for the growth of cells. The floating medium (extracellular) was collected and stored at -20^{0}C. The intracellular floating medium was collected after the lysis and centrifugation of the cell pellet. Then the cell residues were thrown away. After calculating the intracellular and extracellular mixture, the primary enzyme was not seen in the intracellular mix, whereas it is there in the extracellular mixture. With this, we can say the values changed in just 15min. With this result, it is concluded that in the controlled cell sample, PLA_2 expression was more (100%) when compared with the coated cells (-20%) (Fig. 3.10). Finally, we can summarise that ethyl acetate extract of mace can control the PLA_2 formation in the breast cancer cell lines.



Graph 4. Percentage of PLA_2 activity after treating with the ethyl acetate extract of Triphala through *in situ* analysis.

4.8 In silico results:

X-ray crystallography gives specific structures of different proteins by Molecular Docking. Collection of different types of $sPLA_2$'s was taken from the Protein Data Bank for docking with identification number as PDB ID: 1DB5. From the aimed proteins, we can take out ligands in co-crystallized form and water molecules in the

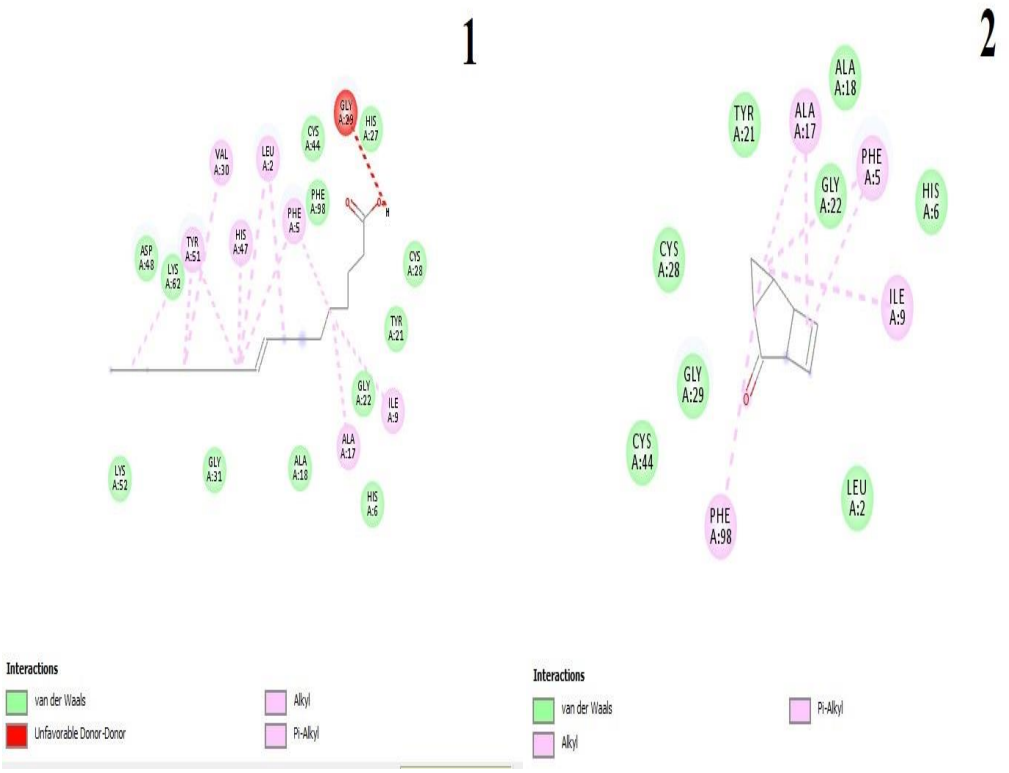
Argus lab, and from the chem office (Cambridge, UK), beneficial ligands were prepared. The energy is minimized to the square root means, the value is decreased to 0.001Kcal/mol. It is calculated by using molecular machines. So, for docking, only that type of ligands and receptors are used, which can reduce energy. GEMDOCK (Generic Evolutionary Method for Molecular Docking) kit used for docking, and in this empirical scoring method used for calculations (A.Rajaram *et al.*, 2015; M.Y.Jinn *et al.*,2004 &J.M.Yang *et al.*,2004). We can use many tools for visual interaction as AutoDockVina (Trott O, Olson AJ*et al.*,2010), PyRx (Wolf LK *et al.*,2009), Discovery studio 2020 or PyMol were used (M.Y.Jinn *et al.*,2004, J.M.Yang *et al.*,2004 and D.Seeliger *et al.*,2010).

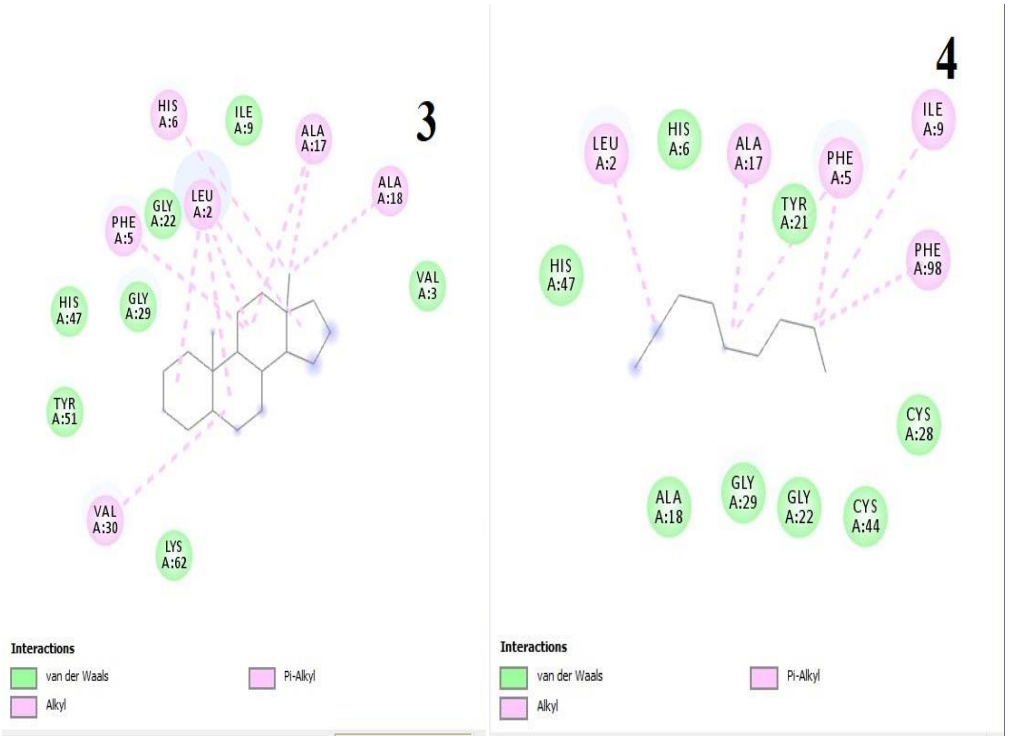
The various active sites of residue amino acid were bonded with a high rate TRP 12 (-95.5282K.cal/mol), according to *in silico*. The active sites of amino acid residue are GLY-22, HIS-47, CYS-44, ALA-18, GLY-29, HIS-6, CYS-28, ALA-17 and PHE-5. TRP 01 (-81.3528K.cal/mol) is the second-highest which will bind to the residue of an amino acid at different active sites of CYS-44, ARG-7, and TYR-21, GLY-29, LEU-2, VAL-3 and HIS's-6. These two may have the controlling capacity of sPLA2 (1DB5) with their more binding capacity and excellent reaction capacity.

Virtual tools, and AutoDock Vina are the docking software's, which indicates the controlling ability of sPLA2 which increases or decreases. We can fix the time for docking, and the values should be maintained in a table (Table 15). The 3D graphical structure of protein bonded with the ligand is shown in a Table.

Table. 15 Docking scores of Triphala compounds against human secretory PLA_2 enzyme (1DB5)

Ligand	Total Energy	VDW	H Bond	Aver Con Pair
trp 01	-81.3528	-72.217	-9.1359	22.45
trp 02	-52.1727	-43.673	-8.5	34.4444
trp 03	-77.0953	-70.095	-7	24.6667
trp 04	-43.5533	-43.553	0	28.5556
trp 05	-58.7965	-45.272	-13.525	27.9
trp 06	-52.9255	-52.926	0	30.9091
trp 07	-70.0985	-51.099	-19	22.4667
trp 08	-74.1342	-68.159	-5.9751	26.5625
trp 09	-73.7015	-69.049	-4.6523	23.8095
trp 10	-80.4446	-78.983	-1.4616	23.7619
trp 11	-68.2214	-62.877	-5.3443	18.4545
trp 12	-95.5282	-83.263	-12.265	25.6364


1
GLY A:29
HIS A:27
CYS A:44
VAL A:30
LEU A:2
PHE A:98
PHE A:5
HIS A:47
TYR A:51
ASP A:48
LYS A:62
CYS A:28
TYR A:21
GLY A:22
ILE A:9
ALA A:17
LYS A:52
GLY A:31
ALA A:18
HIS A:6
Interactions
van der Waals
Unfavorable Donor-Donor
Alkyl
Pi-Alkyl
2
ALA A:18
TYR A:21
ALA A:17
PHE A:5
GLY A:22
HIS A:6
CYS A:28
ILE A:9
GLY A:29
CYS A:44
PHE A:98
LEU A:2
Interactions
van der Waals
Alkyl
Pi-Alkyl


3
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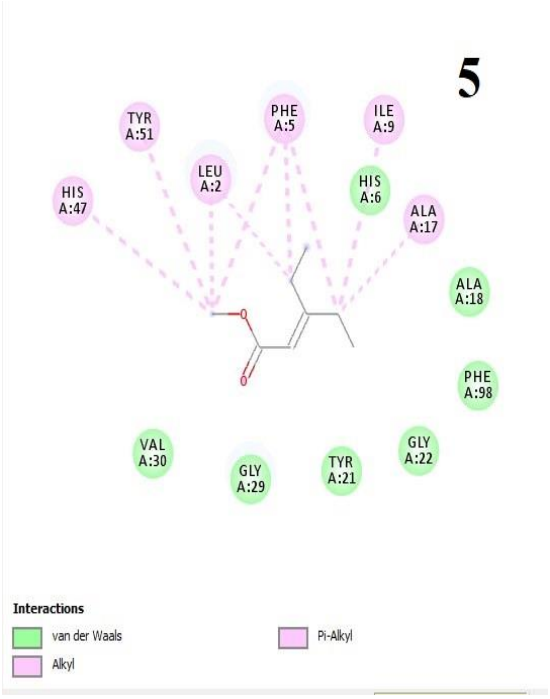
ILE A:9
ALA A:17
ALA A:18
LEU A:2
GLY A:22
PHE A:5
VAL A:3
HIS A:47
GLY A:29
TYR A:51
VAL A:30
LYS A:62
Interactions
van der Waals
Alkyl
Pi-Alkyl
4
LEU A:2
HIS A:6
ALA A:17
ILE A:9
PHE A:5
TYR A:21
PHE A:98
HIS A:47
CYS A:28
ALA A:18
GLY A:29
GLY A:22
CYS A:44
Interactions
van der Waals
Alkyl
Pi-Alkyl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ILE A:9
LEU A:2
HIS A:6
HIS A:47
ALA A:17
ALA A:18
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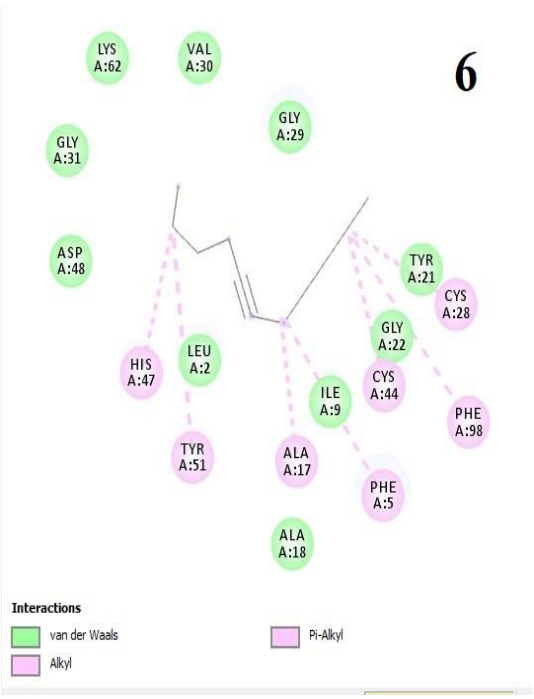
GLY A:22
Interactions
van der Waals
Pi-Alkyl
Alkyl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GLY A:29
GLY A:31
ASP A:48
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CYS A:28
GLY A:22
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LEU A:2
CYS A:44
ILE A:9
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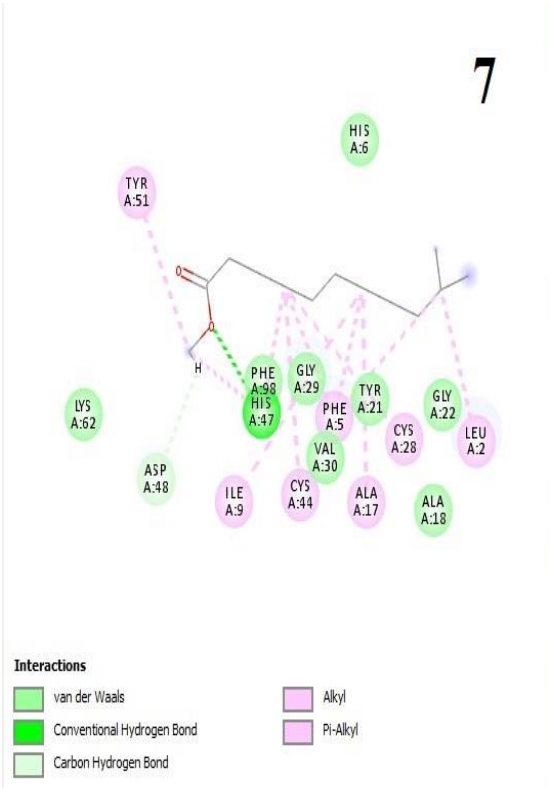
Interactions
van der Waals
Pi-Alkyl
Alkyl


7
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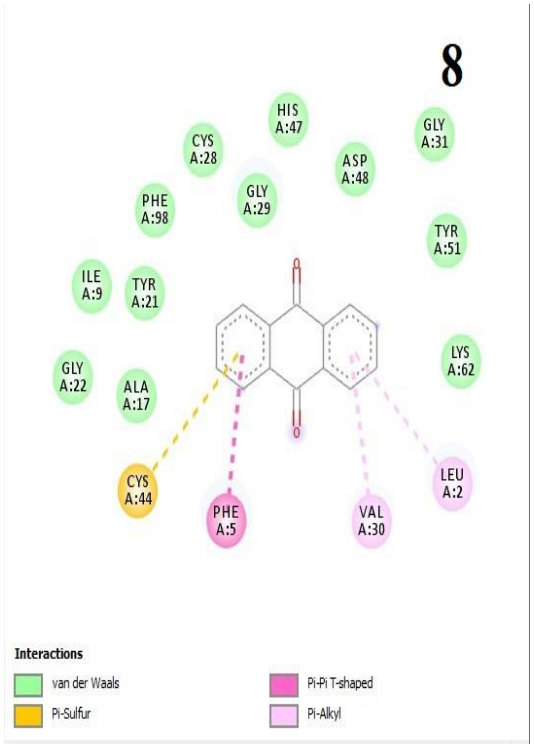
TYR A:51
PHE A:98
GLY A:29
HIS A:47
PHE A:5
TYR A:21
GLY A:22
LYS A:62
CYS A:28
LEU A:2
VAL A:30
ASP A:48
ILE A:9
CYS A:44
ALA A:17
ALA A:18
Interactions
van der Waals
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Alkyl
Pi-Alkyl


8
HIS A:47
GLY A:31
CYS A:28
ASP A:48
PHE A:98
GLY A:29
TYR A:51
ILE A:9
TYR A:21
LYS A:62
GLY A:22
ALA A:17
CYS A:44
LEU A:2
PHE A:5
VAL A:30
Interactions
van der Waals
Pi-Sulfur
Pi-Pi T-shaped
Pi-Alkyl

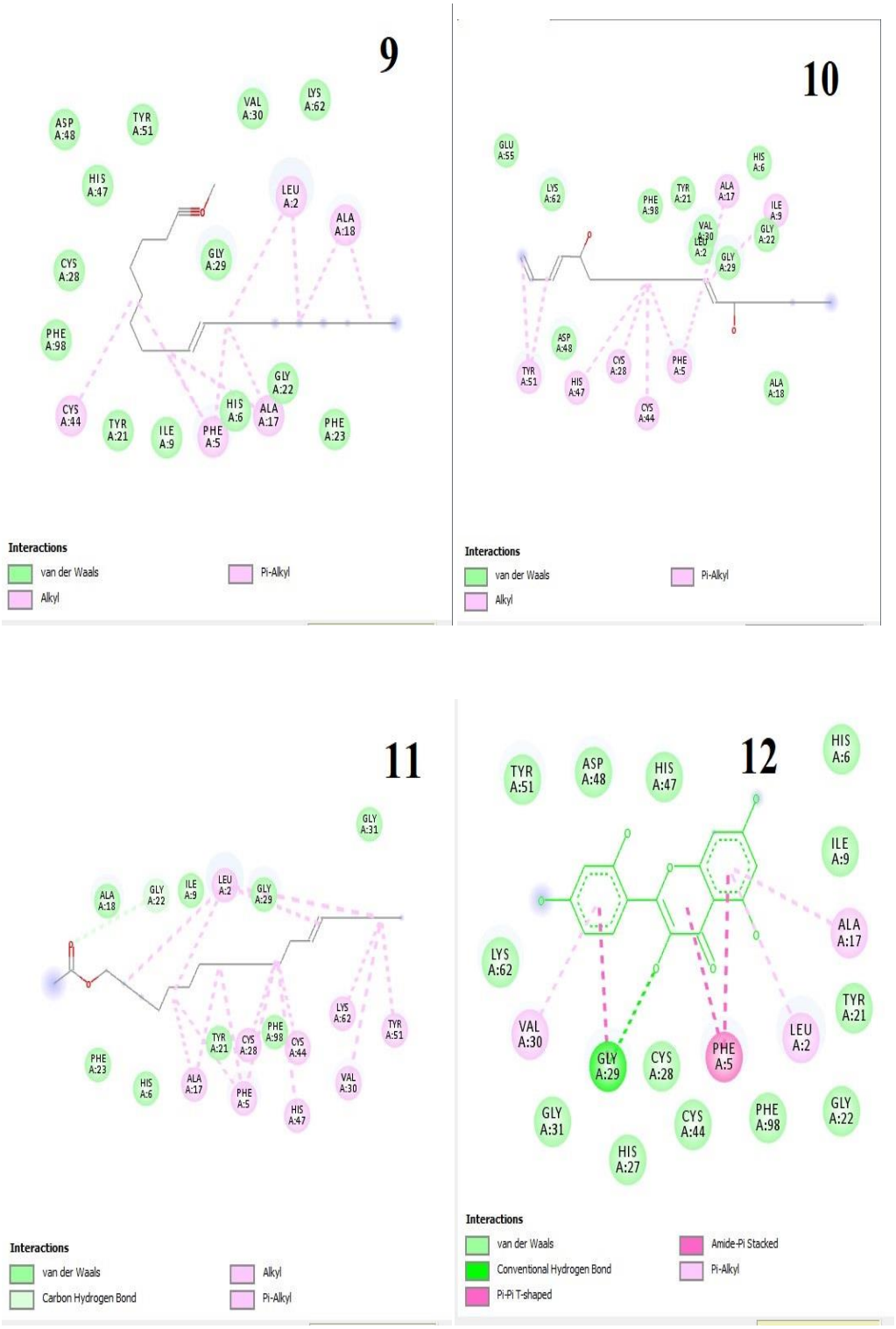


Fig. 25 The 2D diagram shows the interaction between ligand and sPLA$_2$(PDB ID: 1DB5).

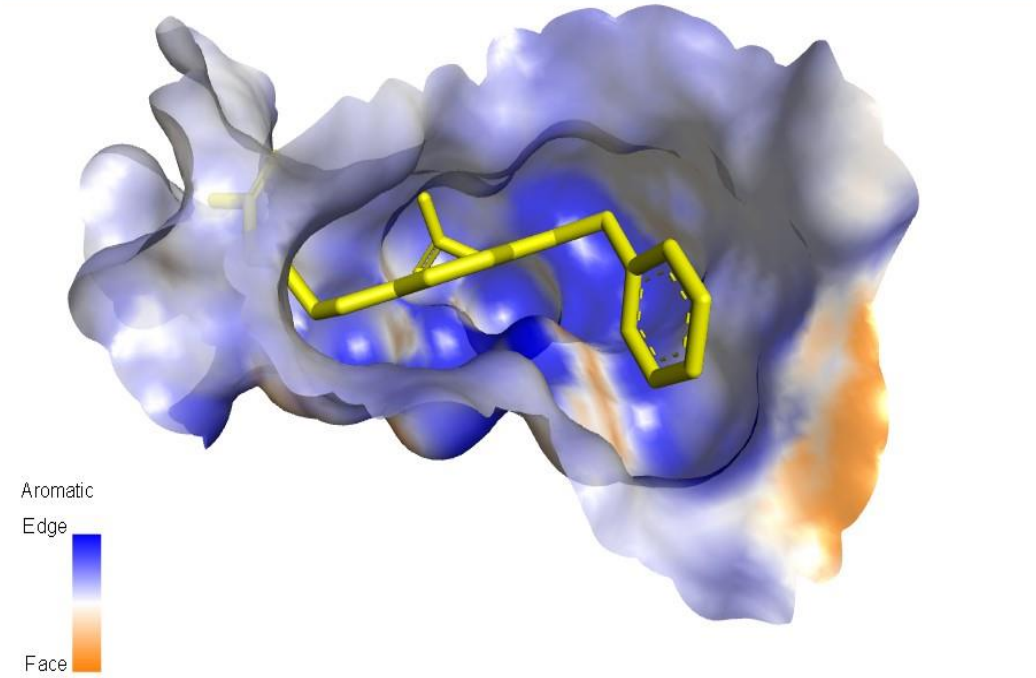
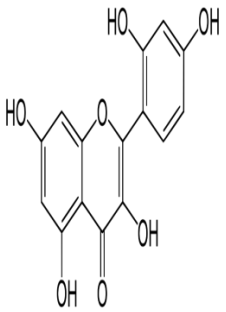
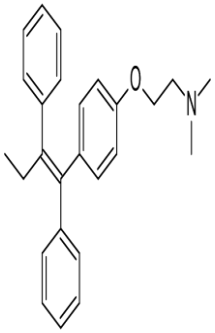
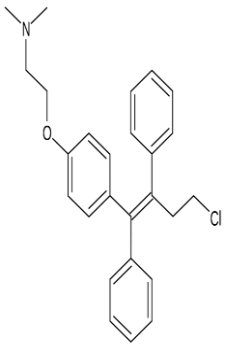
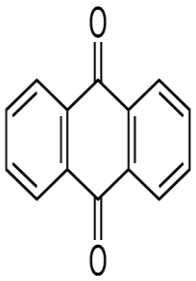
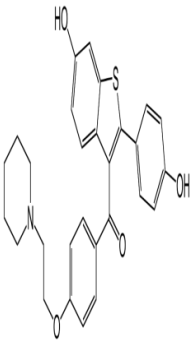


Fig. 26 Surface view of the protein human sPLA$_2$ (light purple) having elemicin (light shade aera) in the active site region.

4.9 ADME/Tox:

Through the docking results, I can say that Raloxifene and Morin are having good binding capacity between ligands and receptors (2iok). The docking value for Raloxifene and Morin are -100.7.36 and -94.8419, respectively. The docking value represents the binding energy of ligand and receptor to give a specific shape. With this, we can see the complete behaviour of the compound. With Morin also we can control cancer cell growth vigorously in the human breast, as it won't give any side effects as chemical drugs (proved in the 3'D and 2'D view of these compound).

Table 15.1 Different lignans with Human estrogen receptor

				
Raloxifene (Ligand)	**Tamoxifen** (Ligand)	**Toremifene** (Ligand)	**9,10-anthraquinone** (Ligand)	**Morin** (Ligand)

Human estrogen receptor

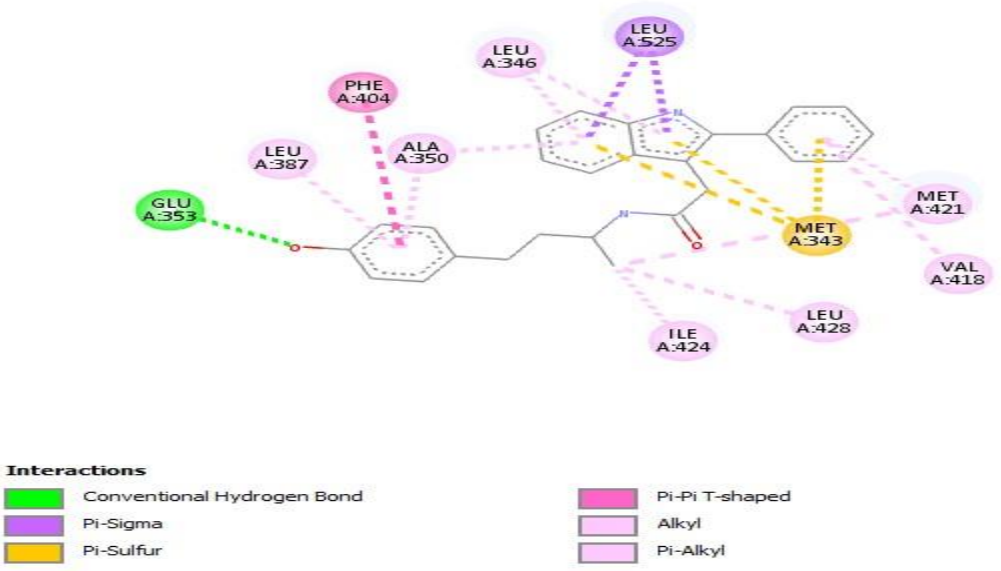


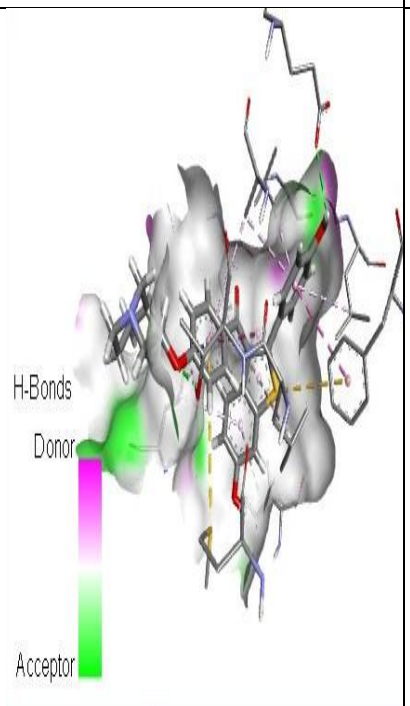
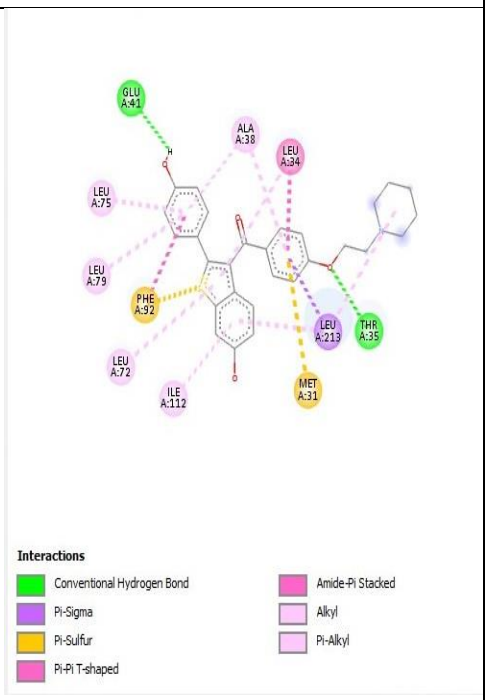
Figure 27. Docking Results of different lignans with Human estrogen receptor (2iok)

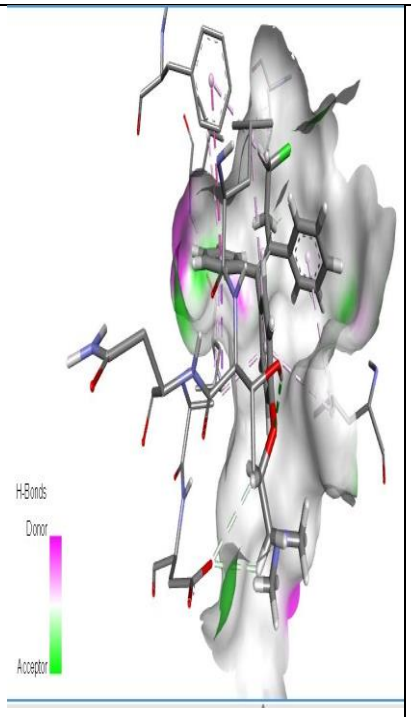
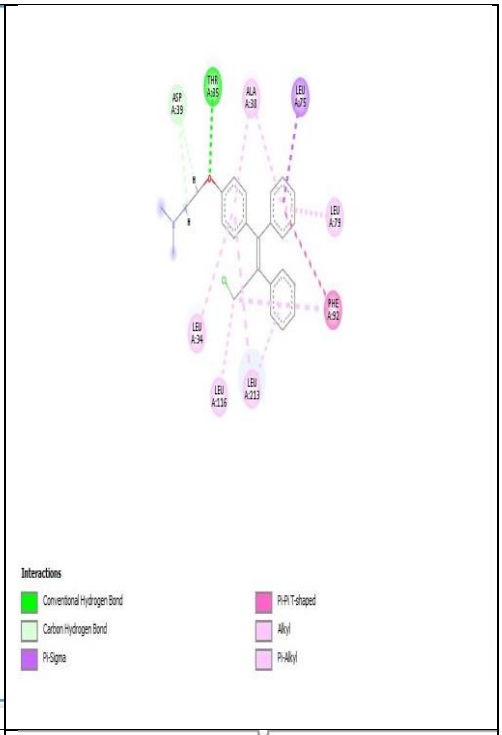
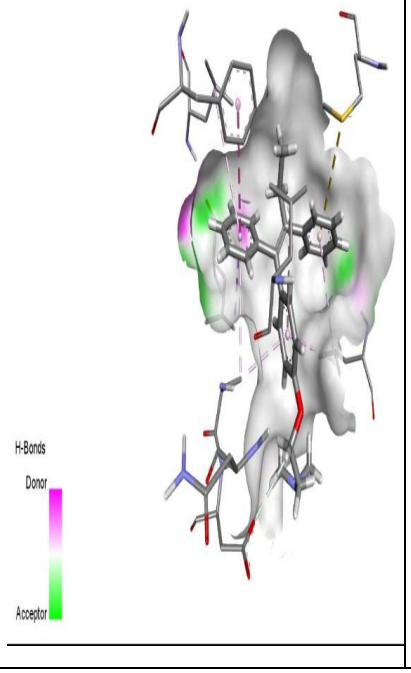
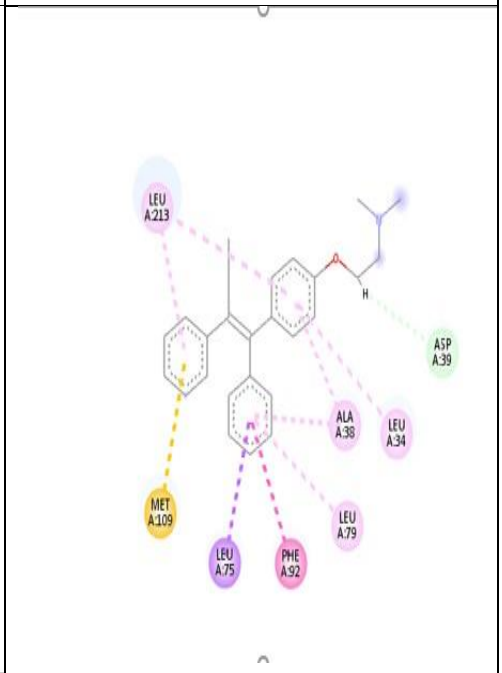
Table. 16 Docking score with 2iok protein

Docking Score with 2iok Protein

Protein	Ligands			
	Compound	**Energy**	**VDW**	**H Bond**
2iok	Raloxifene	-100.736	-96.7115	-4.02425
(Human estrogen receptor)	Toremifene	-87.1032	-84.827	-2.27624
	Tamoxifen	-88.6979	-88.6979	0
	9,10 anthoquenon	-80.2181	-76.7181	-3.5
	Morin	**-94.8419**	-72.3625	-22.4794

VDW: Van der Waals, H-bond: hydrogen bond Elec: Electrostatic information

Compound	3D view	2D view
1. **Raloxifene**		

2. **Toremifene**		
3. **Tamoxifen**		

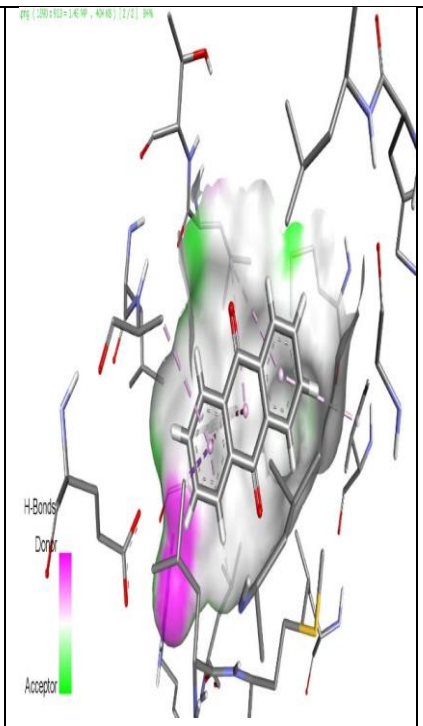
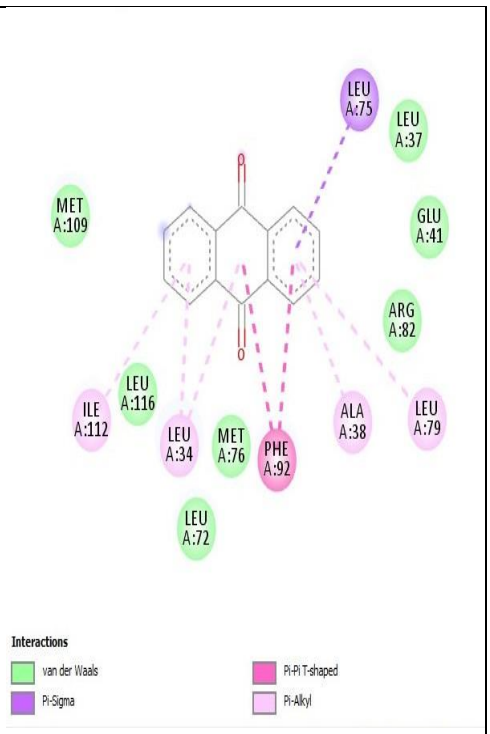
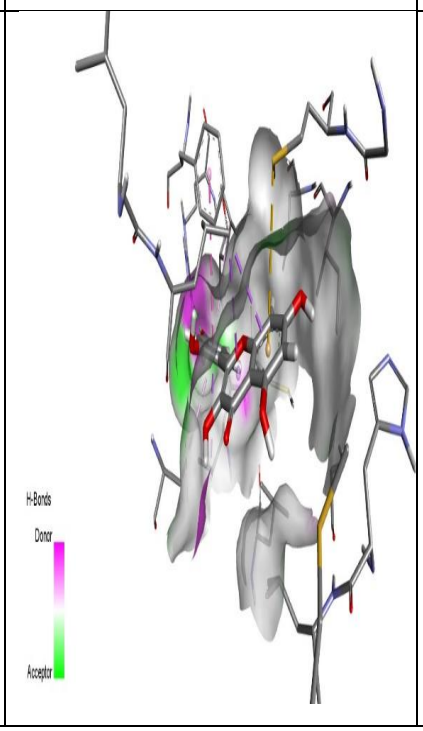
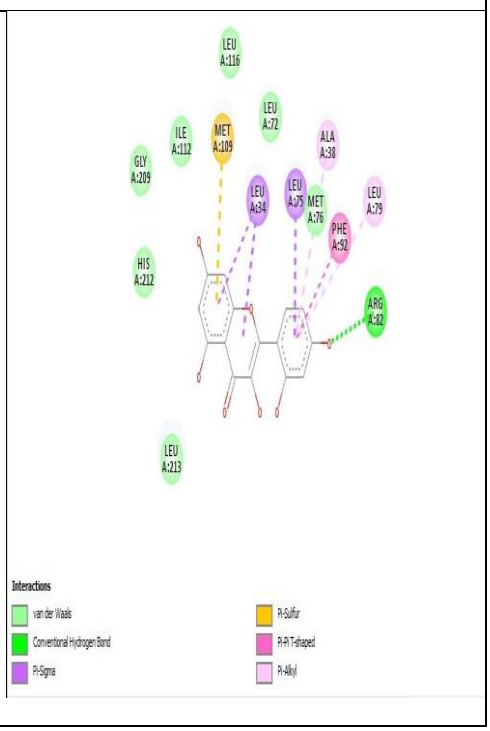
4. **9, 10 anthra-quinone**		
5. **Morin**		

Figure 28. After Docking 3D & 2D structures of different ligands interact with protein (2iok)

4.9.1 ADME/Tox:

When we are using some high dosage drugs in lieu to control or treat the disease, these drugs are giving some side effects. Now, as the awareness is increased, we want to avoid at least some side effects that are caused by these drugs.

Through the ADME/T test, we can easily identify how much of it is absorbed, distributed in the body, the metabolism of the drug, the quantity of drug excreted from the body and the toxic level. Nowadays, ADMET screens are used in labs to know the structure of the compound initial (strong) and later stages (weak) and make it easy to test some important compounds.

A perfect drug should have five important pharmacokinetic properties (PK). They are absorption of the drug completely by the gastrointestinal system, explicitly distributed to the active site, metabolised so the action should not be stopped immediately. The drug should be removed from the body completely.

Finally, we need to know the toxic level of the drug to the organs of a specific individual.

By SwissADME, software the drug sample we took here are also tested for all these 5 tests, giving good results. Table 3.2 shows the comparative study of the drugs based on ADME/T and their values.

According to those values, we can say Morin has some good values. The toxic levels and metabolic levels are satisfactory.

Table. 17 *In silico* results of ADME/Tox

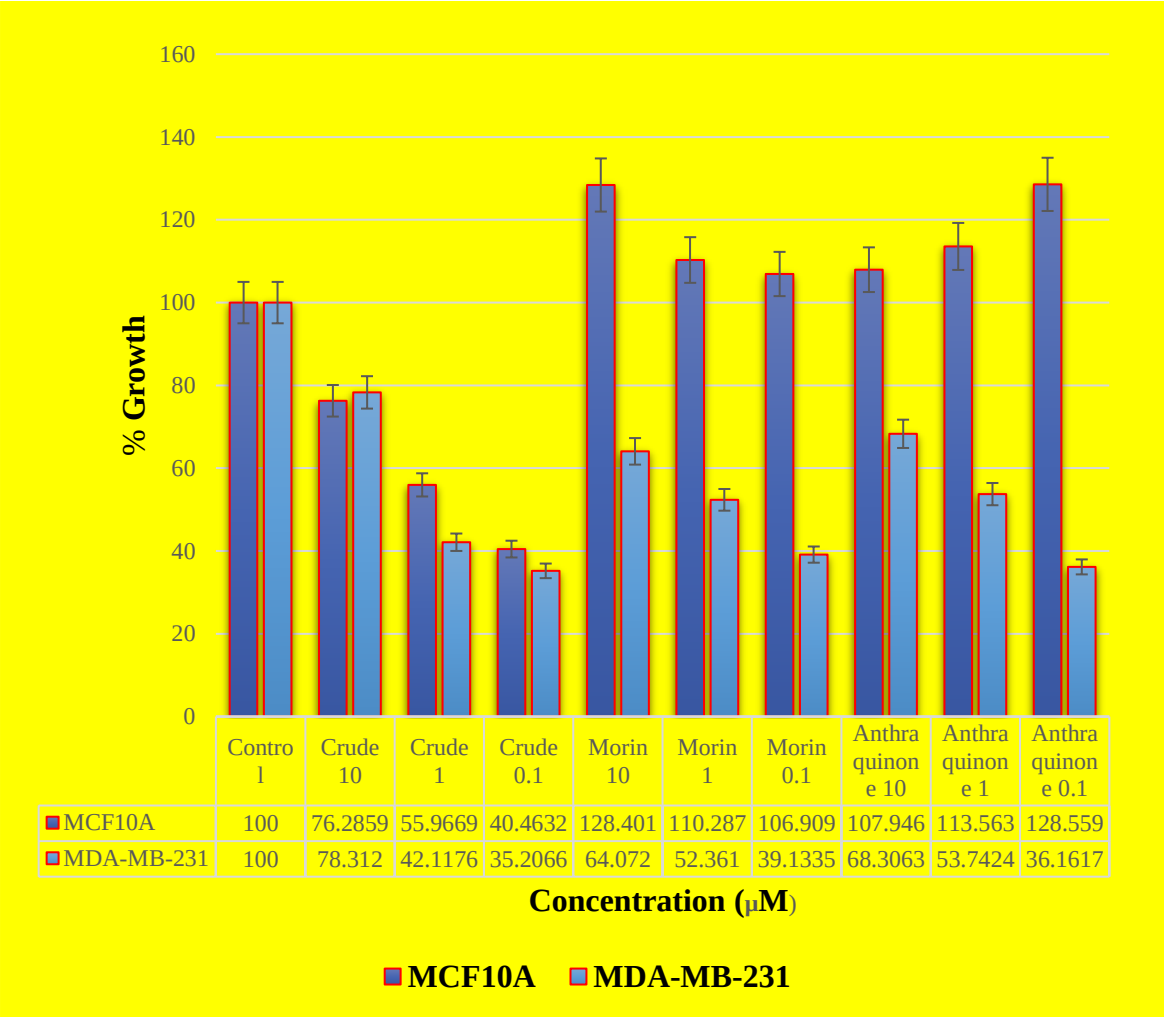
Molecular docking and profiling experiments of *in silicon* ADME/Tox					
Compound	**Raloxifene**	**Toremifene**	**Tamoxifen**	**9,10 anthraquinone**	**Morin**
Formula	C28H27NO4S	C26H28ClNO	C26H29NO	C14H8O2	C15H10O7
Molecular weight	473.58 g/mol	405.96 g/mol	371.51 g/mol	208.21 g/mol	302.24 g/mol
Number of heavy atoms	34	29	28	16	22
No. aromatic. heavy atoms	21	18	18	12	16
Fraction of Csp3	0.25	0.23	0.23	0	0
No. of rotatable bonds	7	9	8	0	1
Num. of H-bond acceptors	5	2	2	2	7
Num. of H-bond donors	2	0	0	0	5
Molar Refractivity	141.21	124.52	119.72	59.75	78.03
TPSA	98.24 Å²	12.47 Å²	12.47 Å²	34.14 Å²	131.36 Å²
Log Po/w (iLOGP)	4.07	4.7	4.64	1.94	1.47
Log Po/w (XLOGP3)	6.09	7.2	7.14	3.39	1.54
Log Po/w (WLOGP)	5.69	6.22	6	2.46	1.99
Log Po/w (MLOGP)	3.21	5.3	5.1	1.86	-0.56
Log Po/w (SILICOS-IT)	6.52	6.5	5.99	3.56	1.54
Consensus Log Po/w	5.12	5.98	5.77	2.64	1.2
Log S (ESOL)	-6.61	-6.76	-6.59	-3.82	-3.16
Log S (Ali)	-7.93	-7.28	-7.22	-3.79	-3.91
Log S (SILICOS-IT)	-8.3	-9.52	-8.92	-5.25	-3.24
GI absorption	High	Low	Low	High	High
BBB permeant	-1.038	1.273	1.329	0.372	-1.18
P-g p substrate	Yes	Yes	Yes	No	No
CYP 1A2 inhibitor	No	No	No	Yes	Yes
CYP 2 C19 inhibitor	Yes	Yes	Yes	Yes	No
CYP 2 C9 inhibitor	No	No	No	No	No
CYP 2 D6 inhibitor	Yes	Yes	Yes	No	Yes
CYP 3 A4 inhibitor	No	Yes	No	No	Yes
Log Kp (skin permeation)	-4.86 cm/s	-3.66 cm/s	-3.50 cm/s	-5.16 cm/s	-7.05 cm/s
Lipinski	yes	yes	yes	yes	yes
Ghose	No	no	No	yes	yes
Veber	Yes	yes	yes	yes	yes
Egan	Yes	no	No	yes	yes
Muegge	No	no	No	yes	yes
Bioavailability Scores	0.54	0.54	0.54	0.54	0.54
PAINS	0 alert	0 alert	0 alert	1 alert	0 alert
Brenk	0 alert	2	1	0	0 alert
Leadlikeness	No	No	No	No	Yes
Synthetic accessibility	3.66	3.18	3.01	2.07	3.25
Fraction unbound (human)	0.119	0.123	0.093	0.136	0.229
Oral rat acute toxicity (LD50)	2.495	2.401	2.285	1.979	2.413

4.10 In vitro anti-cancer effects of AQ and Morin:

On the MCF, 10A cell lines observed the anti-carcinogenic activity of AQ and Morin. By using various concentration gradients of AQ and Morin, the cell lines were grown for 24hrs. This entire process was observed and values were calculated by using an MTT assay. By using various concentration gradients, AQ and Morin acts as cell line controller and has a wide range of activities for controlling cancer. The values of MDA-MB 231 and MCF 10A for controlling growth to 50% (G150) are 50.25 ± 0.228 and 48.2 ± 0.254pM respectively. After this, the MDA-MB-231 cell line study started.

Table 18. MTT results assay

Compounds	MCF10A	MDA-MB-231
Control	**100**	**100**
Crude 10 µM	**76.28593363**	**78.31201836**
Crude 1 µM	**55.96690267**	**42.11756026**
Crude 0.1 µM	**40.46319202**	**35.20659124**
Morin 10 µM	**128.4014331**	**64.07197901**
Morin 1 µM	**110.2874691**	**52.36104279**
Morin 0.1 µM	**106.9094942**	**39.1334645**
Anthraquinone 10 µM	**107.9459183**	**68.30627972**
Anthraquinone 1 µM	**113.5630811**	**53.74241679**
Anthraquinone 0.1 µM	**128.5592425**	**36.16166585**



Graph 5. Fig.48. Effects of Morin and AQ on the proliferation of MDA MB 231 cancer cell lines. The cells were incubated with different concentrations of compounds for 24 h and the cell viability was examined by MTT assay. Concentration-dependent growth inhibition was observed in all the above-tested cell lines. Data are mean ± SEM of three independent experiments (n=3). *P <0.05, #P<0.04 compared with control.

4.11 Morphological effect of AQ and Morin on MDA-MB-231 cells:

For 24 hours, we have to treat MDA-MB-231 and MCF 10A cells with various concentrations of AQ and Morin as 25 and 50 pM. Changed cell structure noted. The growth of cells in standard medium, i.e., without AQ and Morin, is a regular shape with lymph cells in them (**Fig. 29**). But when the cells were cultured in AQ and

Morin medium, the size of the cells was decreased, and the cell wall shows wrinkles on it. The total number of cells were also less when compared with the expected growth of the cells (Fig. 29). Some cells that have developed the outgrowths from the membrane can be easily segregated.

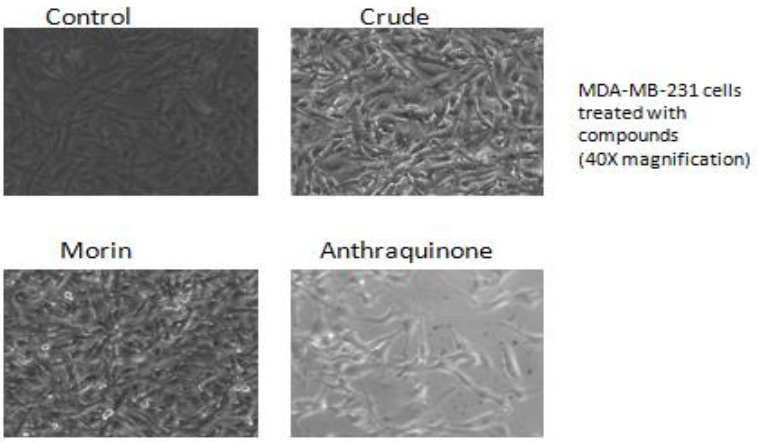


Fig. 29 Growth of untreated (control) MDA MB-231 cells were incubated (5µM) with Morin and Anthraquinone. After 24hrs, we can observe the variations in morphology and the cells were observed with a phase-contrast inverted microscope. Pictures were taken with a Nikon F-601 AF camera (X40).

4.12 In MDA-MB-231cells, DNA fragmentation occurs by AQ and Morin:

In apoptotic cells, a common feature is the fragmentation of DNA. This fragmentation occurs with the biochemical enzymes. AQ and Morin make the occurrence of apoptosis by making fragments of the DNA in a cell. From MDA-MB-231 cells, DNA fragments are collected, some cells (with AQ and Morin or without AQ and Morin) and 1% agarose gel with ethidium bromide of 0.05mg/ml at 5V/cm were exposed to electrophoresis. After taking the picture under UV light, we can see that the DNA strands were broken irregularly at 180-200 bp per fragment **(Figure.30**) The internucleosomal splitting and the task of endonuclease for apoptosis is calculated by the percentage of DNA fragmentation which is proportional to the concentration of the compound. Internucleosomal DNA fragmentation was not there in normal cells (lane 1).

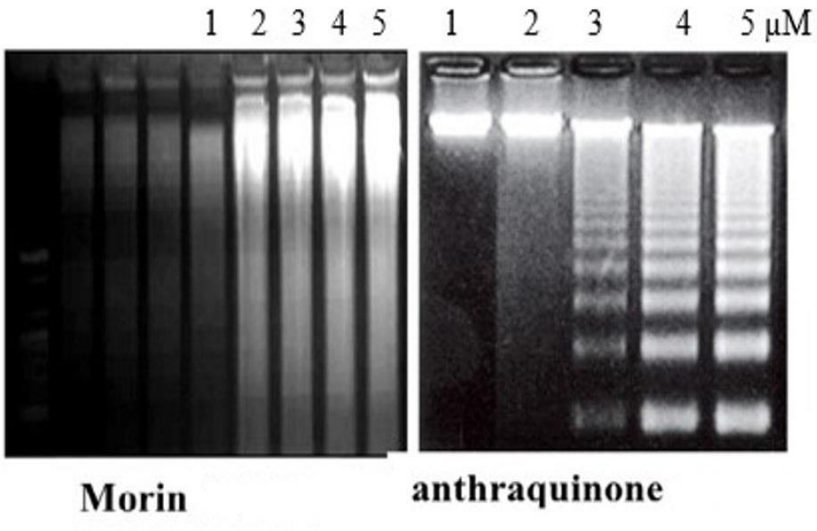


Fig. 30. Agarose gel electrophoresis showing internucleosomal DNA fragmentation induced by Morin and AQ for 24 h in MDA MB 231 cells. After treatment cells were lysed and total cellular DNA was extracted. DNA fragment extracts (8 x 105 cells) were then applied to 1.0% agarose gel containing 0.05 pg/mL ethidium bromide at 5 v/cm. The gel was then photographed under UV illumination.

4.13 Nature of the cell cycle for MDA MB-231 cells with the effect of AQ and Morin: FACS analysis

By flow cytometry, we can know the DNA present in the cells treated with AQ and Morin. In these cells, already apoptosis was generated. The apoptotic cells naturally will lose DNA. DNA collected from the apoptotic and necrotic cell nucleus was stained by PI. This is to see the number of dead cells (Sandström, K et al., 200). More than this, we can also measure the cell lysis by necrosis or apoptosis. Staining with PI is done in two different ways. They are permeabilizing and physiological buffers. PI with a permeabilizing agent can colour the DNA present in necrotic, apoptotic and in living cells PI in physiological buffer stains the DNA in dead cells (i.e., by apoptosis or necrosis). The cells taken for FAC analysis are MDA-MB 231 cell lines. They analyzed for 24 hrs.In crude andAQ and Morin (25 pM). Take the histogram of the cells to measure the content of DNA in them and make a list. By seeing the result, we can say, 29% of cells processed with Morin-25pM andAQ 26% of cells processed

with AQ have reached the G0-G1 of cell division. Whereas the 2% normal cells are not found at this stage of the life cycle.

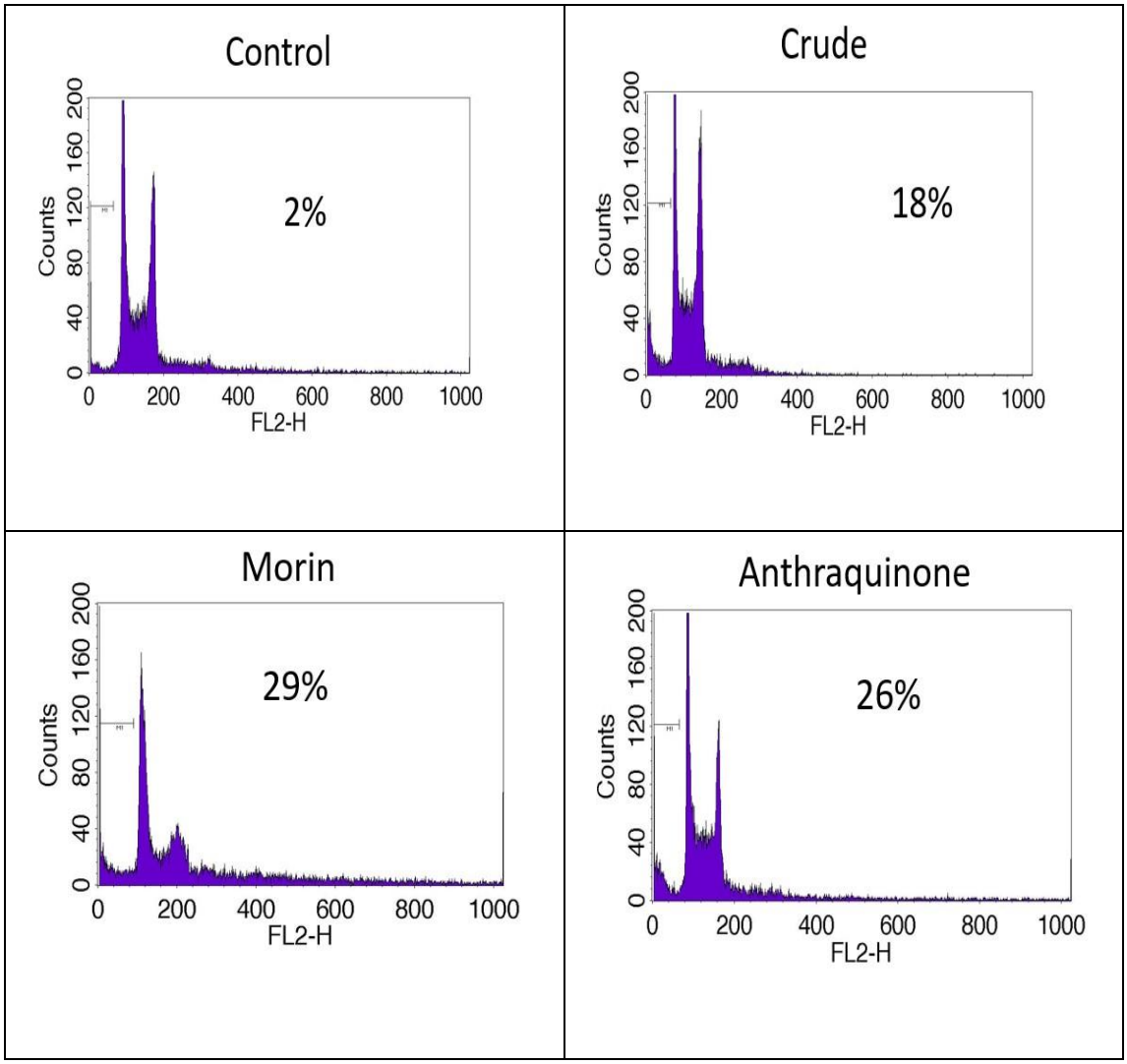


Fig. 31 Quantification of apoptosis in control and Morin and AQ treated MDA MB 231 cells by FACS. MDA MB 231 cells treated with or without Morin and AQ for 24 hrs were fixed and stained with PI and the DNA contents were quantified by flow cytometer. The number of hypo diploid (sub-G0/G1 phase) cells were expressed as a percentage of the total number of cells. Figure shows FACS analysis of (a) control (2%) B) crude (18%) Morin treated 29% and AQ (26%) in MDA MB 231 cells.

4.14 Western blot analysis:

Effects of AQ and Morin **on cytochrome c release and PARP cleavage**

Through the Western blot analysis, we can study the apoptotic mechanism very well. Mitochondria produce Cytochrome C in the cytoplasm, which will initiate the apoptotic pathway. In the respiratory chain, Cytochrome C is a significant protein to be released in the cytoplasm by mitochondria to make the apoptotic pathway active (Liu *et al.,* 1996; Martinou *et al.,* 2000). With the Western Blot analysis, we can see the result of Morin and AQ on Cytochrome C (Fig.32).

The carcinogenic cell lines MDA MB-231 cells were coated with AQ and Morin of 50pM and observed for 0,6,12 and 24hrs. The cytoplasmic parts segregated should be kept on 12% SDS-PAGE for some time. Then place them on the membrane made of nitrocellulose and antibodies of cytochrome C. We can analyze the cytochrome C action on it very quickly. From the data collected, we conclude that in lane one at 0hrs, cytochrome C is not found in the cytoplasm of the cell. As time passed, the presence of cytochrome C was increased in the cells and reached the maximum at 24hrs.

In DNA repair and apoptosis, we can use nuclear enzymes such as PolyADP Ribose Polymerase (PARP). During apoptosis, 116kDa PARP divided into 85 and 23 kDa.PARP antibodies and AQ and Morin of 50pM were coated on MDA-MB-231 cells for 0, 6, 12 and 24hrs time and observed the divided PARP bit of 85kDa and normal 116kDa PARP. From Figure.32, we can say that 85kDa formed by splitting PARP increases with the time intervals. At 0hrs, only the undivided 116kDa of PARP protein is seen.

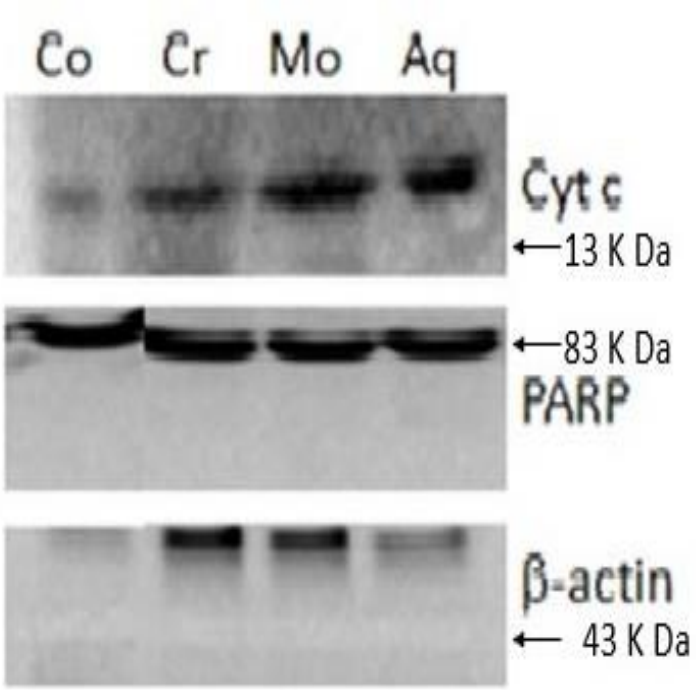


Fig. 32. Through western blot, we can see cytosolic cytochrome c is more in treated cells than controlled cells.

4.15 Changing Bcl-2 / Bax ratio:

The Bcl2 and Bax ratio is vital for cells to survive as these proteins tightly bond to the membrane of mitochondria. By the Western Blot analysis, we can see the difference in the aspect of Bcl2 or Bax cells coated or uncoated with AQ and Morin of 50pM for 0, 6, 12 and 24hrs. Inline 1, we can see Bcl-2 protein with a high concentration for normal or uncoated cells. Inline 2, 3 and 4 Bcl-2 protein decreases when coated with AQ and Morin 50pM along with the time intervals (Fig. 33).

But the level of Bax was constant. So, with this analysis, we got a changed ratio for Bcl2/Bax proteins.

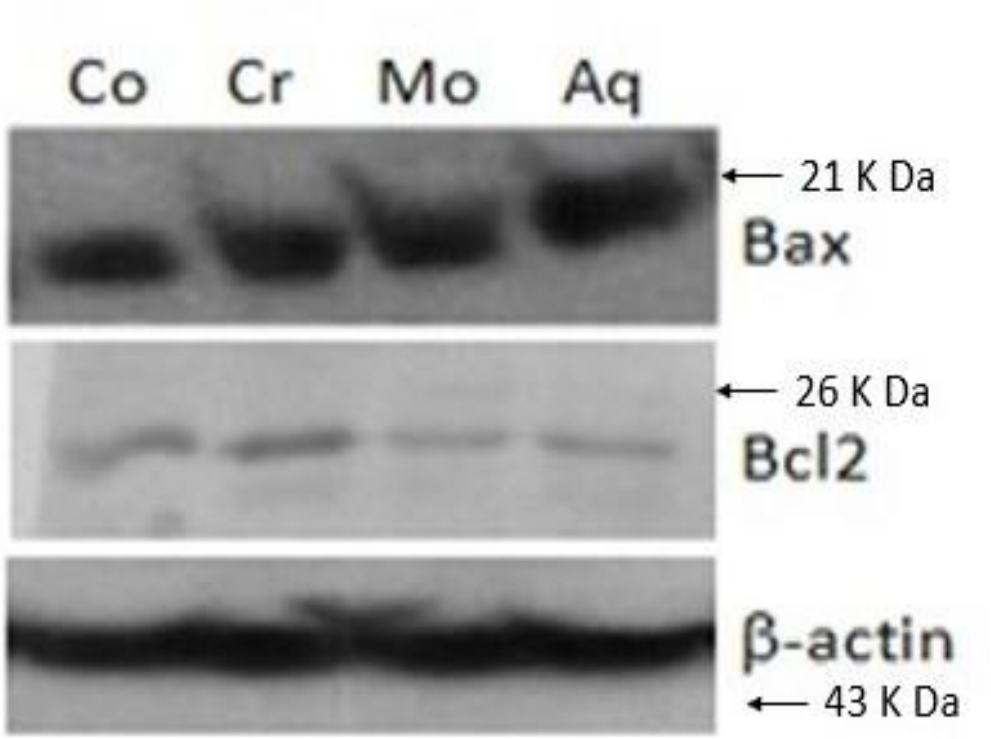


Fig. 33 Immunoblot analysis of Bax&Bcl-2 expression in MDA MB 231 Breast cancer cells treated (5 µM) with Morin and anthraquinone. (AQ and Morin**)**

4.16 Stress protein expression

MDA-MB 231 is a stress response protein present in the matrix of mitochondria. It is produced by the mitochondria to control the stress in the organism. When mitochondrial functions are not seen as regular, then apoptosis attaches with it in releasing Cytochrome C in the cytoplasm or decreases the transition capacity of the mitochondrial membrane. Finally, we can say MDA-MB 231 is related to apoptosis which was initiated by AQ and Morin. As in Fig. 34, protein MDA-MB 231 is more in the cytoplasm of the cells, which were coated with Morin and AQ 50pM in lines 2 to 4. Inline 1, normal cells were present without protein MDA-MB 231.

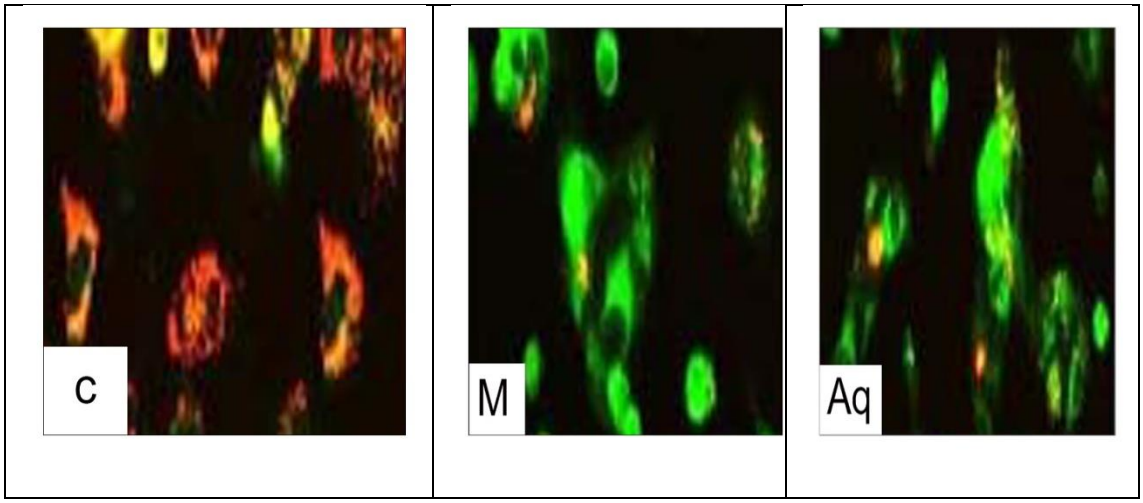


Fig. 34 Mitochondrial membrane potential assay (MMP assay) using JC-10 assay kit, (a) Control cells (MDA MB 231) (b) Morin and (c) AQ

4.17 Nuclear Morphology

In this experiment, we can see DNA is bonded with Hoechst stain under the fluorescent microscope. AQ and morin (5µM) start showing condensed nucleus with bubble. This is the indication for the development of dead cells in MDA MB 231 breast cancer cells (shown by white arrow) in Fig 35, and normal cells are circular.

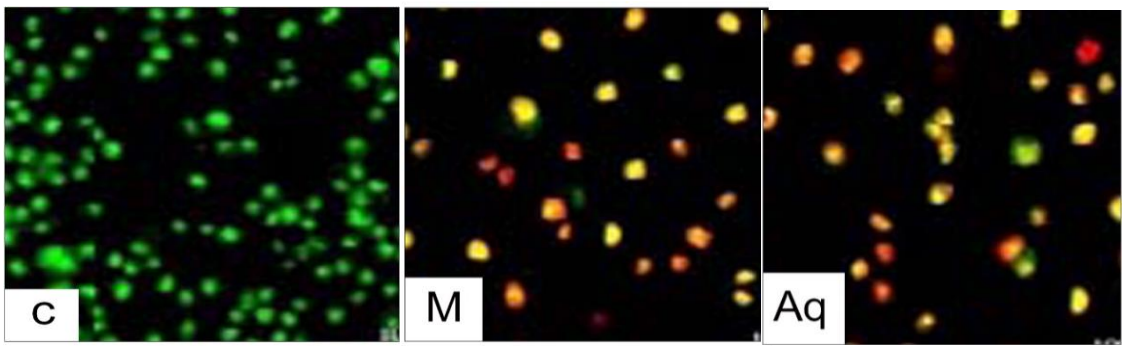
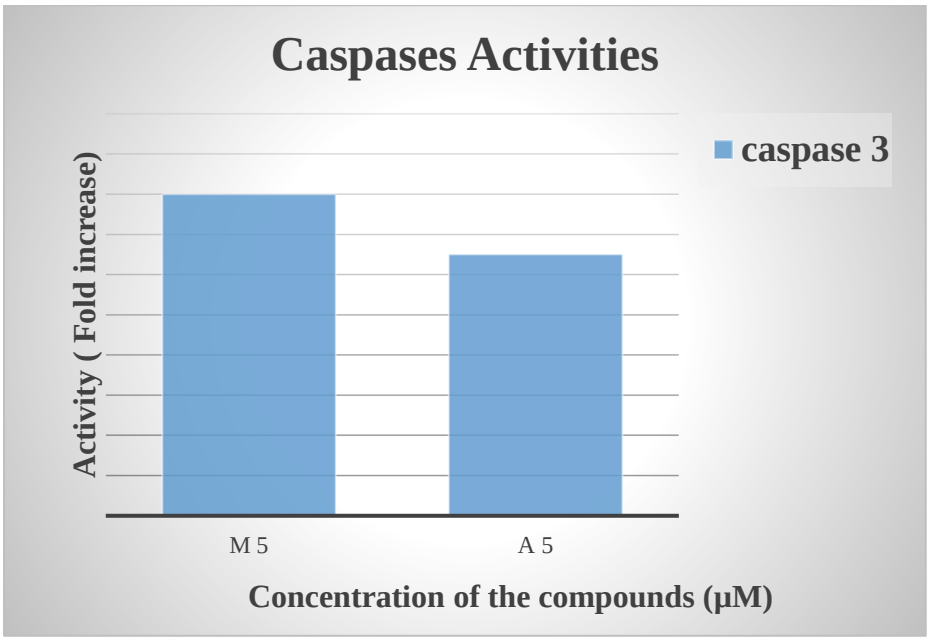
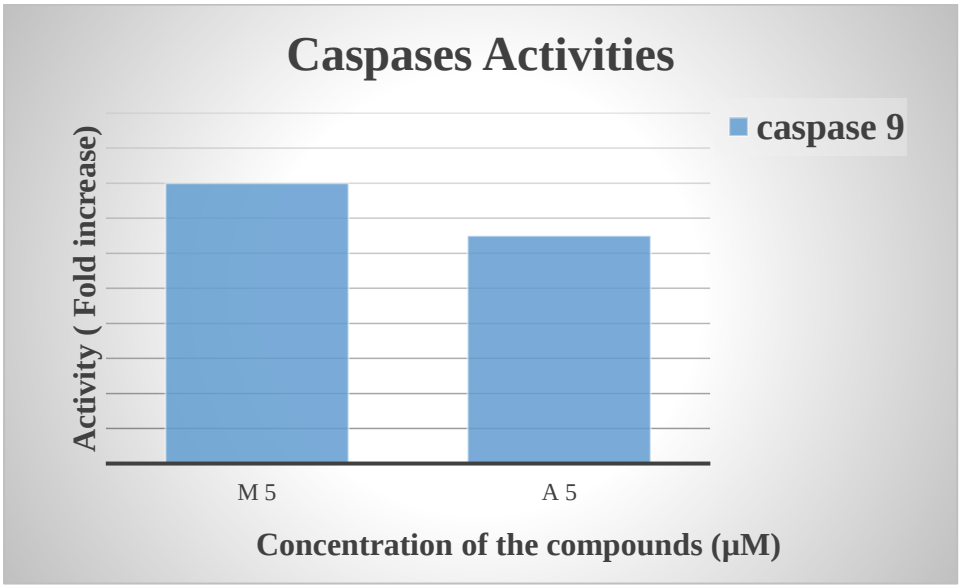


Fig. 35 Nuclear morphological changes in untreated (control) and treated cells (5µM) of breast cancer cells were observed under the fluorescent microscope.

4.18 Effect of Morin and AQ on caspases activity in breast cancer cells;

Morin and AQ-treated MDA MB 231 breast cancer cells were highly active in all Caspases 3 and -9 relative to control cells. The impact on caspase 8 was not seen in both morin and AQ. Caspase activation is characteristic of induction of apoptosis.





Graph 6. Elevation of caspases-3 and 9 activities in Morin and AQ -treated MDA MB 231 breast cancer cells

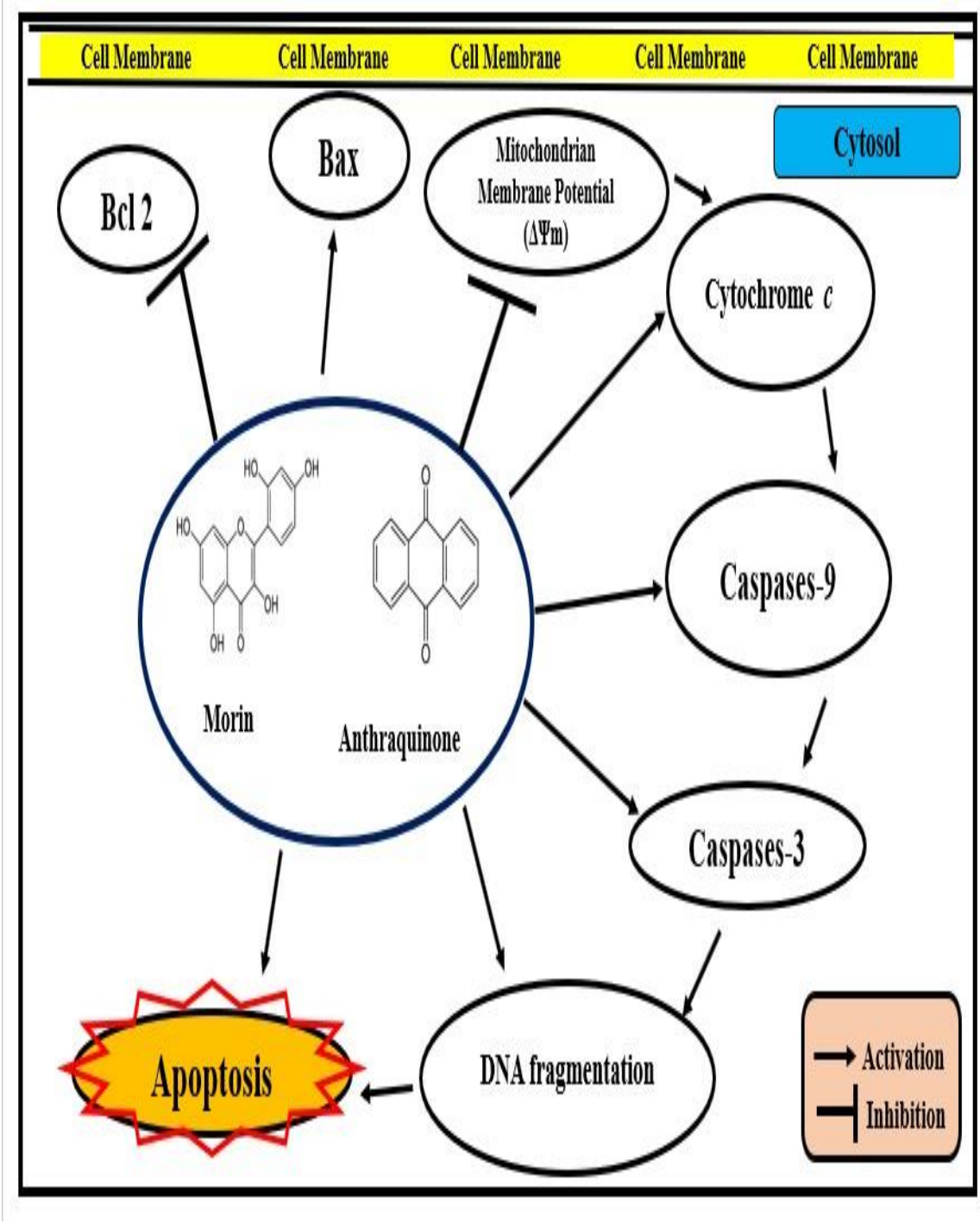


Fig. 36 Diagrammatic representation of the effect of Morin and anthraquinone on cancer cells which leads to death

CHAPTER – 5
CONCLUSION

Anti-cancer potential of Morin (M) and 9,10-Anthraquinone (Aq) of Triphala are the combinations of three plants (*Phyllanthus emblica, Terminalia chebula, Terminalia bellirica*), against Human Breast Cancer cell line MDA-MB-231 in specialized conditions investigated. The pericarp contains considerable amounts of the terpenoid compounds used in the studies. Screening of the chemicals naturally present in the fruit wall such as flavonoids, alkaloids, glycosides, besides terpenoids. The anti-cancer compounds of Triphala fruit powder showed cytotoxic potential for cancer cells in the human breast. These cells are MDA-MB 231 cell lines. Morin (M) and 9,10-Anthraquinone (Aq) controlled the cell division and number of cells at the G0 and G1 phases. These compounds encourage cell death in the breast cell line MDA-MB 231 and intercalated DNA.

The two compounds also exhibited the properties of DNA double-strand break and DNA fragmentation. Morin (M) and 9,10-Anthraquinone (Aq) compounds showed eightfold increased caspase cascade action in breast cancer cells. The anti-cancer compounds of the present study, Morin (M) and 9,10-Anthraquinone (Aq) were found to possess the inhibitory effect on lactate dehydrogenase enzyme and induce loss of cell membrane integrity. Morin (M) and 9,10-Anthraquinone (Aq) also inhibited cyclooxygenase enzyme activity and PARP enzyme activity. Hence, it is concluded that Morin (M) and 9,10-Anthraquinone (Aq) of Triphala constituents could control the human Breast cancer cell in vitro.

CHAPTER – 6
SUMMARY

From times immemorial, the usage of traditional medicines is there in many countries. In India, traditional medicine usage is there from 4000 B.C. onwards; later, this was known as Ayurveda. They were collecting plant extracts for treating several diseases. Many people, like monarchs, too learnt the medicinal system and practised it.In the olden days, medicines were used with rationalism.i.e. genuine knowledge was certain, which was based on the use of reason, logic or in relation to the present-day experience. With the advent of science, there is ample study on medicinal plants and their products in treating and controlling various infections. The plant product does not have any side effects and aresafe for mankind. Herbal medicine has its limitations and is not appropriate in all situations. Modern medicine often results in the short-lived treatment of a symptom rather than the removal of the disease. It most certainly does not boost immunity; constant and unchecked usage of drugs reduce the body's response rendering them ineffective and cause long term side effects. Hence herbal medicines need to be accepted by a developing country like India.

In highly evolved organisms, different enzymatic pathways were found, which play different roles in their metabolism. The arachidonic acid pathway is one of them, which is an essential metabolic pathway in organisms. It includes cyclooxygenase (COX), lipoxygenase (LOX) and phospholipase A_2 (PLA_2) and some metabolites like prostaglandins (PG), leukotriene (LT) and lysophospholipid (LPL). In various diseases like asthma, arthritis, irregular bowel movement, acute respiratory distress syndrome, inflammations in different organelles, atherosclerosis and types of carcinoma, the physiological process associated withthe AA pathway also. The important aspect of the AA pathway is in controlling indefinite cell divisions, irregular growth of cells, increasing cell life span, controlling inflammations and protuberance. The active enzymes of the AA pathway are present in different carcinomas caused by the intake of tobacco, chemicals and UV radiations (those working in labs) are PLA_2, COX and LOX and their products are LT and PGs. These enzymes and products efficiently control the diseased cells. Mostly these are used in

controlling different types of cancers and inflammations also. So many artificial and natural products which can control the AA pathway were invented by many researchers. These products act to control the inflammations and controls the indefinite division of the cells forming tumours. The substances developed from the plants are called natural products. They are ellagic acid, ursolic acid, lycopene, curcumin, genistein, resveratrol, eugenol and 6-gingerol. They are used to control the carcinomas, AA pathways and other different pathways.

Carcinoma or cancer is such a problem where infection or diseased cells divide indefinitely, increasing the mass of cells. With this, it shows protuberance or excess growth in that specific area. Sometimes this infection spreads from one part to another part of the body through blood, serum or sometimes by touch also. Based on the type of behaviour, carcinoma can be divided into two types, malignant and benign. Malignant will spread from one part to another part of the body with uncontrollable cell divisions and growth. Whereas benign carcinoma won't spread from one part to another part of the body. In any living organism, its cell division and all functions occur under control means the process occur in a cyclic manner any number of times and in case any problem occurs it is rectified, but in rare cases when it is not under control, it becomes carcinogenic. Cancers may occur by many factors like tobacco smoking, radiations, with some enzymatic actions etc. This affects the normal behaviour of the gene to become an oncogene and adverse action of tumour-suppressive gene. Some proteins also encourage cancer formation like COX, LOX, MAP kinases etc. In COX and LOX pathway (together called eicosanoids), metabolites formed is Arachidonic acid. It will increase the growth in a cell, irregular divisions and breaking the cell cycles, which leads to the formation of malignant cancer. The activity of PLA_2 is controlled by some high-power medicines containing hydrocortisone, which controls inflammation and cell division effectively. But these chemical medicines have some side effects when used for a long period of time. Those cells also acquire survival against those medicinal compositions over time. To eradicate this dilemma, now we are taking ayurvedic medicines, which does not have any side effects.

In this present thesis, "**Phospholipase A_2 inhibitory activity of Triphala plants constituents for their applications in the treatment of inflammatory and oncology diseases"**, we are concerned to treat cancer cell lines and inflammations with the extracts taken from plants, which have anti-cancerous activity and can be measured by apoptotic assay. Here, I have taken three different fruits from three different plant species which have ayurvedic medicinal characters in them. Individually these fruit extracts act differently, but when combined together, it controls the inflammations and shows anti-cancer behaviour. The plant species are *Phyllanthus emblica, Terminalia chebula and Terminalia bellirica* are called Triphala.

With the ayurvedic information of these plants, this research is mainly focused on the controlling effects on PLA_2 activity in breast cancer cell lines (MDA MB 231). The fruit extracts of Triphala have a natural inhibiting capacity because of the presence of many chemical constituents, but that only Anthraquinone and Morin (AQ and Morin) were taken in the present study. Triphalachurna is taken for cytotoxic studies, PLA_2 controlling activity (*in vitro* and *in situ*) and docking test against PLA_2 (*in silico*) as it contains Morin and anthraquinone.

As per Ayurveda, Triphala is the best medicine to cure bowel movement and for improving the digestion process, detoxifies the body and improve the immunity of the body. The different medicinal uses of Triphala were studied by many people in the PLA_2 pathway.

Phytochemicals present in Triphalachuran are bioactive metabolites like phenolics, flavonoids, alkaloids, glycosides, terpenoids, tannins, steroids, carbohydrates, saponins and proteins. The experiment was initiated by using solvents with less polarity to solvents with high polarity (hexane, ethyl acetate and ethanol as solvents). When compared with other extracts, ethyl acetate gave a high yield of 2.2%.

With the TLC method, it is very easy to know the polarity of the compounds in the extracts. The Triphala extract was added with different types of solvent in TLC and

the results with ethyl acetate or hexane in 6:4 ratios were considered. All the four compounds used in TLC have similar compounds but with variations in concentrations. The inhibitory activity of Triphala extract was assessed by DPPH and free radical assay.

In either of these processes, DCM and ethyl acetate are mixed with IC50 200 and 100µg/ml. This process infers the scavenging potential of free radicals in DPPH and hydrogen peroxide. The harmful effect of the raw extract is observed on the cancer cell lines in the breast. Thus, it is concluded that results depend on different concentrations of extract as it controls the indefinite growth cells. The final value of IC 50 is close to 15.6 µg /ml. PLA_2 assay gives the instant result as to how the extract controls inflammations. Hence it is apparent that DCM & ethyl acetate controls 60% at 200 µg /ml. whereas ethanol and hexane control 50% at 500 µg /ml. Ethyl acetate, when mixed with Triphala powder *in vitro*, has better results than others. Triphala extract was purified and run-on GC_MS chromatography where Morin and 9,10 anthraquinone were separated. It shows the cytotoxic effect on MDA MB 231 cell lines and controls PLA_2 activity in vitro, Thus docking test against PLA_2 (*in silico*) proved.

Morin and Anthraquinone can strongly bond with the target enzyme were proved in silico and in vitro with docking and PLA_2 study, respectively. They relate with the specific enzyme by hydrogen bond interaction and glide energy and compared it with isoeugenol.

DNA fragmentation, flow cytometry and phase-contrast microscopy prove Morin and Anthraquinone as effective anticancer agents on MDA MB 231 cell lines. In the medium of Morin and Anthraquinone of 25 and 50pM, the cells have G0/G2 phase with 31.25 and 52.55%, when compared to 3% in untreated cells. By changing the Bci-2/Bax ratio, Morin and Anthraquinone can initiate apoptosis, increases movement of molecules through mitochondria membrane and outflow of cytochrome c. With the activation of caspase-3, PARP splitting occurs by apoptosis. With this study, we can

say the intrinsic pathway in Morin and Anthraquinone will initiate apoptosis in MDA-MB-231 cells.

The critical results of the thesis are:

- Triphalachuran contains flavonoids, glycosides, terpenoids, and alkaloids, as seen in phytochemical analysis. Terpenoids that effectively controls the growth of cells was isolated by TLC.

- Morin and Anthraquinone were isolated from Triphala extract by the mass spectrograph, which effectively controls the growth of MDA MB 231 cells at 320 nm. Morin and Anthraquinone are cytotoxic compounds.

- Multiple divisions and inflammations in the affected areas are efficiently controlled by Morin and Anthraquinone. Staining by ethidium bromide or acridine orange estimates apoptosis.

- In the infected breast cells, cell growth was ceased by Morin and Anthraquinone. This is estimated by flow cytometry.

- The cells treated with Morin and Anthraquinone efficiently controls the cyclooxygenase enzyme activity, which shows the anticancer nature.

- Molecular docking quickly identifies the protein binding site for $sPLA_2$ extract.

- Thus, the metastatic pattern of breast cancer cell growth in humans can be effectively controlled by the anticancer nature of Morin and Anthraquinone.

FUTURE SCOPE OF THE STUDY

The anti-cancer compounds, Morin (3,5,7,2',5'-Pentahydroxyflavone), 9, 10-Anthraquinone from the fruits (dry) of *Terminalia bellirica, Terminalia chebula*and *Phyllanthus emblica*, respectively. They are found to inhibit inflammation in the body. Morin and Anthraquinone exhibit anti-cancer properties in vitro and the quality of these compounds is determined by their ability to control cancer. The action of these compounds is noted on the specific genes in the cell. Morin and Anthraquinone'santi-inflammatory and anti-cancer activitiesare recommended for drug designing in controlling cancer tumours in humans.

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LIST OF PUBLICATIONS

- "Enzyme inhibition activity both in Vitro and Silico Screening of Triphala Plant Extracts on Phospholipase A_2 (PLA_2)" International Journal of Pharmaceutical Investigation Vol. 11, Issue 2 (2021)

- "Computational investigations and analyses of three main drugs and its comparison with Triphala Plant Constituents as potent inhibitors of Human Estrogen Receptor (2iok): Molecular docking, and in silico toxicity analysis." Journal of Plant Science Today (under 2nd round review)

- "Triphala plant extracts triggered apoptosis in MDA MB 231 human breast cancer cell line" South Africa journal of Botany (under review)

- "Utilisation of the Ayurvedic Drug Triphala in Various Maladies" Journal of Technology & Engineering Science (JTES) ISSN (0975-5289) June 2019 Vol. 2 Issue 2 (A review paper)

- **ICMBDT-2019** (International Conference on Molecular Basis of Diseases and Therapeutics) International conference held from 08.03.2019 -10.03.2019 at Central University of Rajasthan, Ajmer, India. – Oral presentation.

- **NIT Bioengineering 2020** National conference held from 10.12.2020 to 11.12.2020 at National Institute of Technology, Rourkela, India – Oral Presentation.

- **ICIBLS 2020** (International Conference on Innovations in Biotechnology and Life Sciences) International conference held from 18.12.2020 to 20.12.2020 at Delhi Technological University, Delhi, India. – Oral Presentation.

- Attended the two days' workshop on "**cell culture Applications and Techniques**" from 07.09.2018 to 08.09.2018 at Molecular Biology, Department of Chest Research Foundation, Pune India

- Attended the two days' workshop on **"Transcript profiling using Real-time PCR"** organized by the Faculty of Life Sciences and Biotechnology from 01.11.2018 to 02.11.2018 at South Asian University, Chanakyapuri, New Delhi, India

- Attended one-day national webinar on **"Cancer genomics for cancer-free world"** organized by ArulmiguPalaniandavar college, Palani, Tamil Nadu on 08.11.2020

- One day workshop on **"Cloning, purification of recombinant proteins"** from Department of Genetics and Biotechnology, Osmania University, Hyderabad. It was held on 19.03.2021.

- Three-day workshop on **"Genome Editing Technology and Its Application in Form Animals"** held from 17-04-2021 to 19-04-2021 Organized by Division of Innovation and Entrepreneurship, Lovely Professional University Punjab, India.

- A Certificate course **Essentials of Genomics and Biomedical Informatics** offered by ISRAEL-X, Bar-Ilan University, Ramat-gen, ISRAEL. completed on 27thAugust 2021.

- **ICPPB 2021** (International Conference on Plant Physiology and Biotechnology) International Conference held from 10-09-2021 to 12-09-2021 at School of Bioengineering and Biosciences, Lovely Professional University, Punjab, India. - Poster Presentation. **(Best Poster award)**