ESTIMATION OF β CAROTENE AND OTHER SECONDARY METABOLITES IN PLANTS AND ALGAE

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTSFOR THE AWARD OF THE

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CANDIDATE'S DECLARATION

I Garima, Roll Number: 2K20/MSCBIO/07, student of M.Sc. Biotechnology, here bydeclare that the work which is presented in the Major Project entitled -**Estimation of βcarotene and other secondary metabolites in important medicinal plants** in the fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi, is an authentic record of my own carried out during the period from January- May 2022, under the supervision of Dr. Navneeta Bharadvaja .

The matter presented in this report has not been submitted by me for the award for any other degree of this or any other Institute/University. The work has been accepted in SCI/SCI expanded /SSCI/Scopus Indexed Journal OR peer-reviewed Scopus Index Conference with the following details:

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CERTIFICATE

I hereby certify that the project dissertation title "Estimation of βcarotene and other secondary metabolites in important medicinal plants" which is submitted by **Garima, 2K20/MSCBIO/07,** Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the of Master of Science, is a requirement for the award of degree of Master of Science, is a record for the project work carried out by the student under my supervision . To the best of my knowledge, the above work has not been submitted in partor full for any Degree or Diploma to this University or elsewhere. I, further certify that the publication and indexing information given by the student is correct.

Place: Delhi

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ABSTRACT

Plants and algae are emerging as an alternative sources of traditional food to meet the nutrient requirements. These have gained a lot of interest as the major source of nutraceuticals. They are rich in various nutritionally important compounds and possess diverse biological activities for instance antiviral, anti-cancerous, anti-inflammatory, antioxidant, and antimicrobial activity. The plants and marine algae have ample amounts of nutraceuticals compound such as polysaccharides, vitamins, carotenoids, protein, lipids etc. These can prevent from delay and onset of chronic infections. The Spirulina based algal nutraceuticals boost immunity and resist viral infection. In the era when microbes developing resistance to antimicrobial drugs, algal compounds can be an alternative of antibiotics. Algae and plants have convincing characteristics to be a boon for industrial applications such as biodiversity, renewable and biosynthesis of various beneficial compounds.

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CHAPTER 1

1.1 INTRODUCTION

The project focuses on the qualitative and quantitative estimation of flavonoids and Steroids from five different plants. The present project also focuses on the extraction of beta-carotene from the carrot. The value of plants is well known to us. The work also represents the study of the importance of secondary metabolites derived from higher plants. The secondary metabolites are the chemical compounds produced by the plant during secondary metabolism. These also protect plants from biotic and abiotic stress. Secondary metabolites are majorly divided into three groups Phenolics, Terpenes, N and S containing compounds. Compounds belonging to these groups are used in drugs to cure various ailments. These possess various biological activities such as antiviral, anti-obese, anti-cancerous, anti-inflammatory and antimicrobial. The value of plants is well known to us. Plants have always been the traditional remedies against various ailments. Nowadays, interests are growing in natural therapies. Plants have many valuable compounds such as flavonoids, carotenoids, steroids, alkaloids and tannins. Out of these flavonoids are the largest group among phenolic compounds with various biological activities[1] They absorb harmful UV radiation and prevent plants from microbes and insects. Steroids are also a therapeutically important group of secondary metabolites. Steroids comprise therapeutically important bioactive compounds. These also play an important role in plants. The five medicinal plants used for the study of steroids and flavonoids are *Moringa oleifera, Catharanthus roseus, Hibiscus rosa Sinensis, Murraya koenigii, and Kalanchoe pinnata.*

On the other hand, the microalgae that are used in the food supplement or nutraceuticals are *Porphyridium, Spirullina, Chlamydomonas, Anabaena, Crypthecodinium, Synechoccus, Nostoc, Chlorella, Hematococcus* etc.[2] *D.salina* majorly utilised in nutraceuticals for commercial production of compounds such as soft gelatine capsules of beta carotene by companies like Carlson and Nature's plus[3]

The aromatic secondary metabolites are Flavonoids, tannins and phenols. The double bond in the ring absorb UV radiation. These also give rise to different color of plants. The flavonoids majorly possess antioxidant activity. Some of the flavonoids also possess antibacterial, antiallergic, anti-inflammatory properties [4]

The steroids derived from plant contain one – five membered and three six-membered rings. Some glycosides are linked with steroids like digitalis from Digitalis purpurea which is enough to treat heart disorders. Steroidal saponins are also essential precursors of manufacturing drugs[5]

A medicinal plant is any plant in which any of its part, contain compounds or precursor of substance that can be used for therapeutics. These are rich in secondary metabolites and essential oils. The use of medicinal plants usually focuses on the treatment rather than prevention of disorders. The WHO also confirmed the use of green medicine can help 80% of the global population. More than 78% drugs belong to antimicrobial and anti-cancerous drugs These herbal medicines are affordable and easily available[6] The WHO also made the strategies and guidelines to recognize the need for and importance of medicinal plants. With the increasing interest in herbal medicine, the first drug was used in the middle 19^{th} century. The bioactive compounds derived from plants possess antioxidant, antibacterial, antiviral, anti-inflammatory, and insecticidal activity[7]. Due to increased drug resistance, there is a need to

search for new sources of the drug molecule to treat various diseases. The annual production of herbal medicine is also very high[8]

The secondary metabolites can be extracted from the plants naturally but due to the regional and environmental limitations, the commercial production is highly affected. Due to the various therapeutic benefits, these can help in maintaining the good health in the time when the major reason of diseases are poor life styles[9][10]

Carrots are enriched with the precursor of vitamin A, which is beta carotene. Beta-carotene provides yellow, red and orange pigments to the carrot. It is a sub-group of carotenoids and possess antioxidant properties. The antioxidant activity prevents from various disorders such as heart disease, cancer, neurodegenerative disease, and eye cataracts[11]

The indicatory test has been done which shows the presence of flavonoids and steroids in the fresh leaves, shade dried and sundried leaves of five different plants. The quantitative estimation of flavonoids has also been done that are used to calculate the amount of the flavonoids present in the plant. On the other hand, the quantitative estimation of beta carotene has been done using three different red carrot and the results are compared.

The objectives of the projects are

1. **To determine the presence of steroids and flavonoids in the following medicinal plants.**
    1. *Hibiscus rosa sinesis*
    2. *Moringa oleifera*
    3. *Murraya koiniggi*
    4. *Catharanthus roseus*
    5. *Kalanchoe pinnata*
2. **Quantitative estimation of flavonoids in the following medicinal plants**

1. *Hibiscus rosa sinesis*
2. *Moringa oleifera*
3. *Murraya koiniggi*
4. *Catharanthus roseus*
5. *Kalanchoe pinnata*

3. Quantitative estimation of β-carotene in Daucus carota

1.2 ORGANISATION OF THESIS

The following thesis title as **Estimation of Nutraceutical compounds in Plants and Algae** is a reviewed information gathered from various research and review articles. The thesis focuses on the estimation of metabolites in different medicinal plants and quantitative estimation of beta carotene in carrot. The thesis also contain information on the effects of the metabolites on human.

The chapter 1 that is the introduction of thesis briefs about the plant secondary metabolite, applications in therapeutics and their biosynthesis.

The chapter 2 is review literature that contains a broad knowledge about the medicinal plants and their therapeutic role. It summarizes all the plants that used in the project for qualitative and quantitative estimation . It also include the solvent based extraction method and the principle of UV – vis spectroscopy used for quantifying the different bioactive compounds. It discusses the importance of the compounds in nutraceuticals and future prospects.

The chapter 3 is a proposed methodology for the project and briefs about the extraction process, estimation techniques that can be used for detection of flavonoids , steroid and carotenoids.

The chapter 4 contain Results, Discussion and conclusion part.

CHAPTER 2. REVIEW OF LITERATURE

Phytotherapy is an emerging concept for maintaining good health. Nutraceutical originated from plants are highly recommended. Nutraceutical possess various therapeutic activities . Some of the medicinal plants used in nutraceuticals are discussed below.

2.1 *Moringa oleifera*

M. oleifera is tradition local herb used in medicines. “The common name of Moringa are Mulangay, Drumstick tree, Sajna, Kelor, Marango, Mlonge and horseradish tree. The hierarchical division of Moringa oleifera is “Kingdom: Plantae, Division: Magnoliphyta, Class: Magnoliopsida, Order: Brassicales, Family: Moringaceae, Genus: Moringa, Species: M. oleifera”. *M. oleifera* is a potent source of nutraceuticals and about all the part of it can be used a source of nutrition [12]It consists of carotenoids in leaves, essential amino acids, vitamins, beta carotene, fatty acids and other valuable compounds that can be used in nutraceutical. Common people named it as ‘the miracle tree’ because of its property of its healing properties. This plant is indigenous to many places in the pacific Caribbean, Southeast Asia Arabia and Africa Islands and South America. *Moringa oleifera* is in the sub-Himalayan region of northwest India. This plant is used for treating many ailments such as anaemia, skin infection, blood impurities, cataract, renal, cholera and chest ingestion. Moringa also possess anti-tumour, antioxidant, anti- epileptic, diuretic, hepatoprotective and anti-spasmodic activity.[13]

2.1.2 Anti-inflammatory Effects

Moringa can cure chronic and acute disorders and has been used in medicine for many long years. Studies have also shown that the moringa is effective in healing hypergycemia,

hyperlipidemia and inflammation. The secondary metabolites from Moringa possessing antioxidant, anti-microbial and anti-inflammatory are flavonols and phenolic acids.

2.1.3 Antimicrobial Properties

The plant part of moringa which exhibits antimicrobial properties are leaves, root, stem, ssed coat and root bark. Singh et.al studied the antibacterial property of *Moringa olifera* by kirby baur disc diffusion method using ethanolic extract of moringa leaf. The methanolic and aqueous extract promises the treatment of some specific bacterial infections. It was reported that leaves extract found to inhibit growth of Gram positive bacteria such as *S. aureus* and *E.faecalis* .

2.1.4 Anticancerous properties

It is reported specifically in south asia that moringa has a potential to fight against rheumatoid arthritis, diabetes, cancer and other diseases. Recently it is investigated that moringa has a chemopreventive agents. It reduces progression of tumor and was also reported that the it helps in reducing the number of tubular adenocarcinomas. The presence of fatty acid in moringa also found to modulate programmed cell death in colon cancer. The extract of moringa pod help in protecting from the effects of carcinogens. Also oxidative damage due to PAH 7 , DMBA can be protected by moringa extract and derived saponins.

2.1.5 Antioxidant property

Polyphenols from moringa are known to reduce oxidative damage in cells. In the DPPH free radical scavenging experiment leaf extract showed reduction in the DPPH radicals. The more the amount of polyphenols in the plants more will be the antioxidant activity.

2.*2 Kalanchoe pinnata*

Kalanchoe is also one such genus of plants that are used to cure many ailments. Kalanchoe is a large genus, having colourful flowers. It is a succulent perennial plant with erect herbs and a glabrous stem with purple scales. Kalanchoe belongs to the family Crassulaceae and consists of 1410 species. Apart from their ornamental properties, they do have medicinal applications. They possess a crassulacean acid metabolism photosynthesis pathway that helps them to grow in dry conditions. Based on flower morphology and geography Kalanchoe is mainly divided into three groups, Bryophyllum, Kitchinga and Kalanchoe. There are various phytochemicals present in the leaves of Kalanchoe such as alkaloids, steroids, flavonoids, tannins, saponins, sterols, carbohydrates, and triterpenoids. [14]. Herbal practitioners called this plant a Master herb. *K.pinnata* is the oldest species of Kalanachoe discovered in gardens of Europe and is the most widespread species, now known for its medical properties. Gaind and Gupta first reported the occurrence of flavonoids and rutin in *Kalanchoe pinnata*, *K.brasiliensis* is also known for the same medical properties and biological activities. The most common biological properties possessed by these species are anti-inflammatory and immunomodulatory activities. The immunomodulatory activity is used for healing wounds and skin diseases. Studies have shown that mice infected with *Leishmania amzonesis* when orally treated with aqueous leaf extract of *K.pinnata* help to decrease the size of lesion and parasite load as compared to Glucantime injection.[15]. Conventionally this plant was only used against fever, but now it has been explored for other ailments also[16]. The leaves of this wonder or divine plant possess a class of chemicals called as bufadienolides whose activity is very similar to cardiac glycosides, digitoxin and digoxin. Digitoxin and digoxin are the drugs used during the treatment of heart

failure and conditions related to it[17] It has different ethnomedical uses globally. In Mexico, used to cure eye infections, pimples, and menstrual disorders while in Peru it is used against bacterial infections, cancer, epilepsy, migraine, sores, and inflammation. In India, this plant is distributed all over the country and used for swelling, insect bites, and wounds in the Himalayan region while in Orissa it is used against diarrhoea. In Arunachal Pradesh, it is used to cure urinary bladder stones and is taken in an empty stomach. Moreover, the juice of the leaves is also used against dysentery in Maharashtra and in Karnataka, the juice of the leaves is externally applied over cuts, in order to stop bleeding[18][19]. Recently, it was reported in mice that the aqueous extract of *K.pinnata* can be used against fatal anaphylactic shock.

2.2.1 Antioxidant potential: Antioxidants protect cells from reactive oxygen species which can cause cell damage. In the therapy of cardiovascular illnesses, potential antioxidant activity has a strong association. The leaves had the greatest scavenging effects compared to the stems, and the ethanolic extract had higher total phenolic and flavonoid content than the other extracts. high levels of phenols and flavonoids may explain their strong antioxidative activity. The capacity of phenolics to stabilise radicals through the formation of stabilised phenoxyl radicals by directly scavenging peroxyl radicals explains the inhibition of lipid auto-oxidation [14].

2.2.2 Wound healing potential: In a study by Biswas et.al wound healing in animals was observed with ethanolic extracts of *K. pinnata* and significant reduction in the wound area was observed on day 11 through control treatment [20]. The presence of steroid glycosides in the extract may be responsible for the extract's wound healing abilities. Bufadienolide, a steroidal compound found in the plant as a steroidal glycoside, has been found in substantial amounts in the medicinal plant[21].

2.2.3 Antimicrobial effects: Some common gram-negative and positive bacteria and fungi were inhibited by these phytocompounds. Pattewar et al., (2013) investigated the antibacterial activity of K. pinnata and found that the methanolic extract inhibited bacteria more effectively. Bacteria that cause skin infections can also cause food poisoning, wound infections, osteomyelitis, pneumonia, and other consequences when they enter the body[14]. In a study by [22] at a dosage of 25mg/ml, Kalanchoe pinnata leaf extract (60 percent methanolic extract) was found to suppress the growth of five of the eight microorganisms tested.

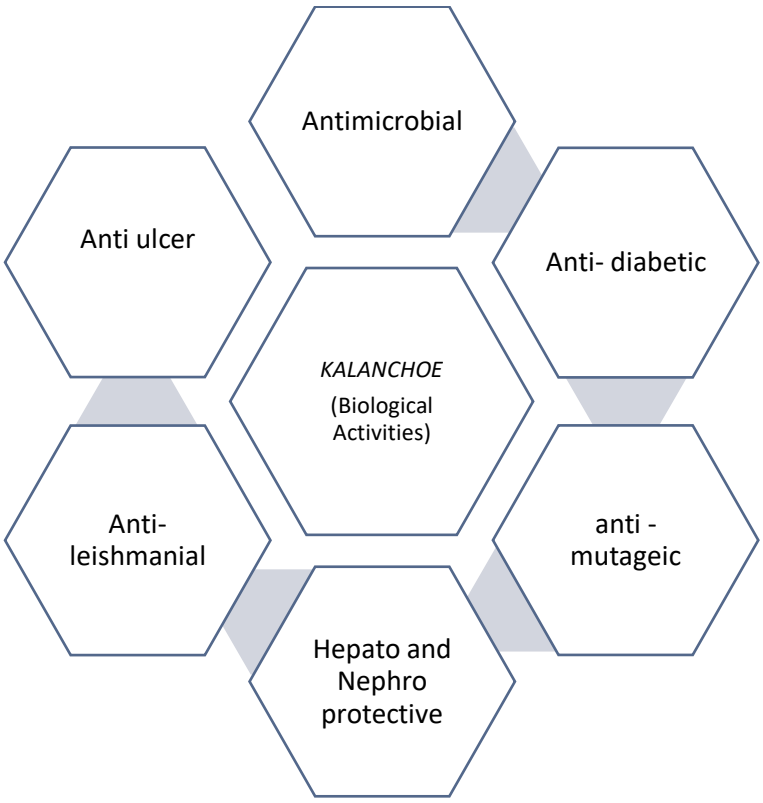


Fig.2.1- Different therapeutic effects of *Kalanchoe* sp.

2.2.4 Leishmaniasis activity potential: Infections caused by Leishmania protozoa are a serious global health issue, with a high endemicity in developing nations. The flavonoid quercitrin is responsible for antileishmanial action. Antileishmanial activity appears to be

dependent on the quercetin aglycone-type structure [23]. Da Silva et al. studied the antileishmanial characteristics of three flavonoids identified in leaf extract (quercetrin, quercetin, and afzelin) in mice against *L. amazonensis* amastigotes and discovered that the oral route was more efficient than other methods [23]. The preventive effect of plants in leishmaniasis may be due to macrophage stimulation of the reactive nitrogen intermediates pathway rather than a direct effect on the parasite [24] In a study by effect of quercetin was studied at clinical trials. Quercetin (16 mg/kg body weight) was given as a daily oral dose. They were able to drastically lower the parasite load and inhibit the progression of Leishmania amazonensis-caused lesions.

2.2.5 Immunosuppressive effects: In a study conducted by on mice it was observed that, an aqueous extract of the leaves inhibits both cell-mediated and humoral immunological responses [25] In vitro, the ability of spleen cells from animals primed with plant extract to proliferate in response to mitogen and antigen was reduced. The capacity of mice to mount a delayed type hypersensitivity reaction (DTH) to ovalbumin was likewise harmed by extract treatment. By virtually eliminating the DTH reaction, the invitro and topical modes of delivery were the most effective[23].

2.2.6 Antidiabetic Effects: The presence of zinc in the plants could indicate that they could be useful in the treatment of diabetes caused by insulin dysfunction. The ethanolic extract of K. pinnata reduced blood glucose levels in diabetic rats. As a result, the serum glucose level drops and glucose tolerance rise. The plant extract also enhanced insulin production from the pancreas [14] [26] [23].

2.3 *Murraya koenigii (Curry tree)*

India is known for its variety of different medicinal plants. The taxonomical classification is Kingdom ; plantae, Division -Magnoliophyta, Class -magnoliospida, Order Sapindales, Family:Rutaceae, Genus-Murraya and Species murraya koengii . it is widely used in spices and the word kari originated from Tamil . Now it is cultivated in different regions all over the world for instance Srilanka , Africa, Australia, China and pacific island. It is enriched with organic compounds with medicinal properties such as phenols, proteins, flavonoids, sterols , terpenoids.[27]

These possess different biological activities as follows

1. Antidiabetic – Murrayacine and murrayazlinine are the phytochemicals which decreases oxidative stress by effecting Paraoxonase 1 activity
2. Anti oxidant -koenigine and mahanimbine are the chemical constituent that increases the gsh amount in the liver and reduce hepatic malondialdehyde in kidney
3. Anticancerous – mahanine and murrayafoline are the compounds derived from curry tree that increases the death of cancerous cell .
4. Wound healing effects – mahanimbine, mahanine, essential oils and mahanimbicine act against the inflammatory cells and also reduces the collagen deposits.

2.4 *Hibiscus rosa -sinensis*

The genus hibiscus contain around 200 species all over the world and they possess a ample amount of bioactive molecule such as triterpene, phytosteroids phenolic compounds. these possess anti-oxidant, cardio preventive and antiproliferative properties. Hibiscus is "generally recognised as safe" hibiscus is native plant of China. It shows various pharmacological activities as follows [28]

1. Antifertility activity – It is reported that the plant shows antifertility activity in rats. Studies have also shown antidertility effect in women also.
2. Antifungal activity- It shows 34.50% mycelial inhibition in Rhizoctonia solani .
3. Antiviral activity- The plant extract found to inhibit the activity of measles virus, coxsackie B2 virus, polio virus.
4. Anti- implantation effect – Various part of the plant has been investigated for studying the effect on reproductive system. The plant extract prepared using benzene showed post-coital antifertility effect in reproductive system.
5. Other miscellaneous activity – Antipyretic, anti-inflammatory , antidiabetic and Antimicrobial activity.

2.5 ALGAE BASED NUTRACEUTICALS

The Ocean covers more than 70% surface area of the earth's surface where a major diversity of marine microalgae resides, enriched in valuable compounds that can be used in nutraceutical. The word "Nutraceutical" come from "nutrition" and "pharmaceutical" coined by Dr Stephen Defelice in 1989. These are compounds that contain one or more dietary components such as amino acids, minerals, vitamins, medicinal herbs, any product taken in diet by an individual, extracts, metabolites or fusion of the ingredients[2][29]. Nutraceuticals not only provide nutrients but also promote treatment and prevention of disease[30]. Food supplements are increasingly in demand as the population is growing globally and algae can serve as potent source of renewable nutrition [31]. Algae are known for decades as traditional food and complementary medicine[32][33]. In ancient time seaweeds were used for preparing Japanese Sushi and Korean food gimbap[34]. Algae is a source of broad range of nutrients such as carbohydrates, PUFA, proteins and trace nutrients like antioxidants, vitamins[31]

2.5 PROPERTIES OF SECONDARY METABOLITES

2.5.1 Properties of Flavonoids

Flavonoids are aromatic secondary metabolites, having a benzene ring that absorbs UV radiations. Because of the absorbance of different wavelengths of visible light, these can give provide different pigments to the plants. Flavanoids are a large group containing various families of compounds approx. 10,000 . Quercetin is the dietary flavonoids present abundantly and has promising antioxidant compounds along with anti-inflammatory and anti-allergic properties. Due to their antioxidant properties, they are also used in the cosmetic, pharmaceutical, nutraceutical and food industries. Based on structure, these are classified into 6 major classes, anthocyanin, flavanones, isoflavanones, flavanols, flavan-3-ols, and flavones [1]

Anthocyanin is a flavonoids that give rise to color of the flower and are also situated in leaves , roots , stem and fruits. These are commonly used in food industries as food additives. Structurally, anthocyanins are anthocyanidins O glycosides. Anthocyanideins are also oxidised coloured pigments but unstable.

Flavanones are formed by isomerisation of hydroxychalchocones. Many plant derived flavanones are bounded with sugar in the form of 7-O-glyosides. One of the mostly studied examples is astilbin that possess potent antioxidant activity. These are also called as dihydroflavones, majorly present in citrus fruits.[35]

Isolation of Isoflavanones are limited to few subfamilies of Leguminosae family. These exhibit estrogenic activity. These are basically derived from flavanones by rearrangement followed by dehydrogenation and the chemical name of these flavonoids are 3-aryl-4H-chromen-4-ones.

Flavonols and Flavones are 2-aryl-3 hydroxy-4H chromen-4-ones and 2-aryl-4H-chromen-4-ones respectively. These are also acquired by dehydrogenation of flavanones. Flavones are the

representative and large class of flavonoids . The most common flavanol is Quercetin, which exhibit various biological properties . Some of the useful flavanols are sinensetin, luteolin, galangin, chrysin, apigenin and isosinensetin.[1]

2.5.1.1 Biosynthesis of Flavonoids

Primary metabolism in plant leads to the synthesis of flavonoids. These are produced by mitochondrial and plastid derived intermediates. These intermediates are then reached to cytoplasm and from there they are incorporated into different part of molecules. It comprises of three rings in its structure in which ring B and chromane ring are formed from phenylalanine aminoacid. Phenyalanine itself is produced by Shikimic acid pathway. The ring A originates from the condensation of 3 molecules of malonyl CoA added as a result of decarboxylation-condensation reaction, initiating flavonoids synthesis.[36] In phenylproponoids pathway, the enzyme that performs the conversion of phenylalanine to cinnamic acid is Phenylalanine ammonia-lyase. This forms C6-C3 structures. Enzyme chalcone synthase catalyzes the formation of the first flavonoids naringenin after the condensation of three units of malonyl-CoA and 4-coumaroyl-CoA. After that, the isomerization of chalcone occurs to form flavanones by chalcone flavanone isomerase. This flavanone formed is an intermediate for the synthesis of all different groups of flavonoids[37] .

2.5.2 STEROIDS

Steroids comprises of therapeutically important secondary metabolites. Steroids and teracyclic terpens are similar in structures but have different biosynthetic pathway. all steroids are derived from S-squalene -2,3-epoxide which is an intermediate compound of acetate mevalonate pathway. Classification of plant steroids is based on their biological activities , structure and taxonomy . There are seven major classes of steroids[38]

Phytosterols are steroids derived from plants. These are major components of plant lipid bilayer , cell membrane and help in maintaining membrane fluidity and permeability. These are ubiquitous in plant kingdom. Due to hypocholesterolemic activity, phytosterols has gained more attention recently. The most common phytosterols present in plants are campesterol, stigmasterol, avenasterol and beta-sitosterol. These steroids are different from cholesterol by an alkyl group at carbon-24. The common source of phytosterol are vegetable oil. Beta sterol and campesterol are present in large amounts in plants[39]These can lowers the blood cholesterol level and also affects the absorption of cholesterol. Moreover, phyterols also possess in vitro anticancerous activity and are known to inhibit progression of cancer cells in human. It has been studied that beta sterol is useful in treating benign prostatic hyperplasia(BPH). Phytosterol derived from the leaves of *Mentha cardifolia* possess analgesic properties[40]

Withinolides is another class of steroids, its function in plants is not well studied but in mammals they show important biological activities. These are steroidal lactones. The plants that contain withanolides are Pysalis angulata, W.somnifera , Dunal (Ashwagandha). These plants are known to be formulated in ayurvedic medicines since 3000 years ago. Dunal alone comprises of over 150 formulations in Unani, Siddha and Ayurvedic medicines. They exhibit antitumor, antimicrobial, immunomodulatory, anti-inflammatory, and anti-leishmaniasis activity[41].

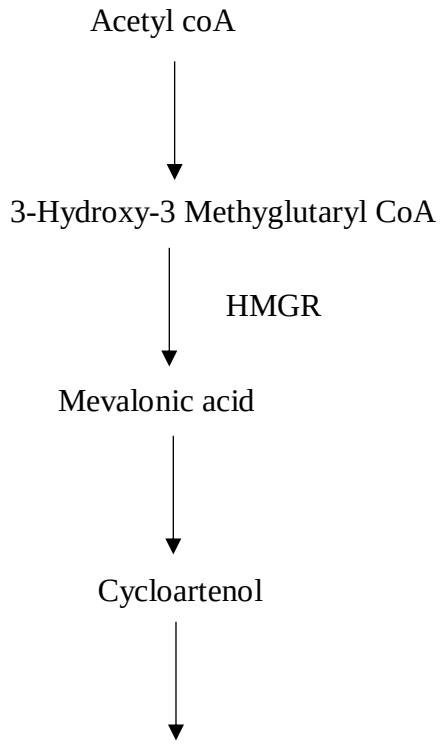
Brassinosteroids are hormones regulating normal growth in plants. The classification of this class done on the basis of carbon number , C-27, C-28 and C-29 substituent group at carbon-17. They have two rings A and B. They play important functions in plant as they prevent plant from different stress like salinity, extreme temperature, drought, heavy metals and herbicidal injury. Moreover, they play a remarkable role in mammals also as they exhibits neuroprotective and antioxidant activities. The first brassinosteroids is named as brassinolide and was obtained

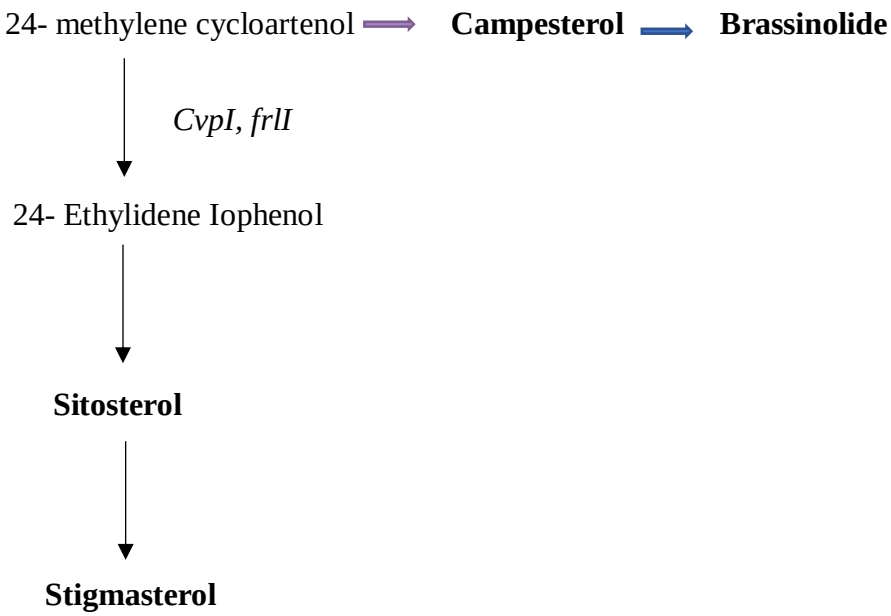
from the pollen grains of *Brassica napus* . due to the expansion of one ring in the structure of these steroids, these are known as steroidal lactone. The three indicative and important groups of brassinosteroids are epibrassinolide, castasterone and brassinolide[42]

Phytoecdysteroids are ecdysteroids and play important role in the development of invertebrates. The plants containing these steroids are *Podocarpus nakaii, Polypodium vulgare , Achyranthes fauriei* . These contain C-28,C-27 and C-29 steroidal skeleton and are formed cis fusion of A and B rings. These are generally polyhydroxylated and contain hydroxyl group at C-14, C-2, C-22 and C-20. Phytoecdysteroids are used by athelets for body building. Apart from this they shows antiarrhythmic, anabolic, hepatoprotective and immunostimulant properties. Moreover, these are safe and are not antigonadotroic, androgenic and thymolytic.[43]

2.5.2.1 Biosynthesis of Steroids

The biosynthetic pathway of steroids is as follows.[40]





2.6 CAROTENOIDS

These are the pigment extracted from algae and possess antifungal, anti-inflammatory, anti-tumour, anti-fungal and anti-oxidative properties. Beta carotene and astaxanthin is the high value commercial source. The pigments are utilized in beverages as food colour, in aquaculture as feed additives, ingredient in pharmaceutical and cosmetics[44][45]

In plants, function of harvesting light and photo protection of photosynthetic apparatus by scavenging the free oxygen species and free radicals is performed by the carotenoids and other pigments such as phycobilin present in plants [46] A large variety of carotenoids have been discovered and largely used as colouring agents in food industries. Some of the commercially available carotenoids are Lycopene, beta carotene, astaxanthine.[47]

Astaxanthin is a non-provitamin that cant be transformed into vitamin A in human body , and ketocarotenoid which is popularly known due to its antioxidant activity because of the presence large number of conjugated double bonds that is 13. and also pigmentation in various dietary supplements and salmon flesh. It has higher antioxidant activity than lycopene and beta carotene.[48] It has a unique compound structure which help it to stay both inside and outside the membrane. Antilipid peroxidation activity is one of its feature as it inhibit lipid peroxidation in biological samples. It shows anti-diabetic activity, protects pancreatic beta cell against glucose toxic effect. It is of great importance to the drug manufacturers as it provides protection against cancer by targeting free radicals [49].

Consumption of astaxanthin doesn't have risk of toxicity.this is available in the market in form of oil, tablets capsule ,syrup ,creams etc[50] .It cause alteration in insulin like growth factor Decrease risk of cancer and cardiovascular diseases.

β- Carotene is a provitamin A carotenoid. These can be converted to retinol and decreases the danger of macular degeneration. It reduces the risk of cancer and obstructs DNA damage and can cause apoptosis in cancer. Commonly used as coloring agents and nutrition supplements Because of its antioxidant property it is used in cosmetics also .

2.6.1 Biosynthesis of β Carotene

A C_5 compound named isopentyl pyrophosphate (Isopentenyl Diphosphate (IPP) (IPP) is precursor of terpenes, isoprenoids, sterols, quinones, carotenoids and phytol of chlorophyll. There are 2 most common independent pathway for IPP synthesis; the malvonatepathway (Mevalonate pathway) (MVA) and non-malvonate i.e. 1- deoxy-D-xylulose-5-phosphate pathway (DOXP). In the DOXP pathway IPP is formed precursors i.e.glyceraldehyde's -3-

phosphate (Glyceraldehyde-3-phosphate) and pyruvate which make use of 1- deoxy-D-xylulos-5-phosphate (DXP) and it is converted to IPP.[51][52]. In MVA pathway, IPP is formed from acetyl coenzyme A mediated by malvonate some enzymes. The DOXP pathway is present in cyanobacteria, the plastids of some animal and land plant, and in some bacteria whereas MVA pathway is found in euglenophyceae , animals , plant cytoplasm and few bacteria [52][53]

Carotenoid contains 8 subunits of IPP. out of 8 three IPPs are utilized to synthesise Farnesyl pyrophosphate(C15) and then 1 IPP is added by geranyl geranyl pyrophosphate synthase (CrtE, GGPS) to produce gernayl geranyl pyropfhosphate (c20). and then by condensation of two C20 compounds catalyse by phytoene synthase (CrtB, Pys, Psy) utilizing ATP to yield the first carotene i.e phytoene (C40)[52][53]. This pathway studied in cyanobacteria and 2 species of green alga . The genes crtB and crtE are very similar in sequence from bacteria to land plants .Phytoene to Lycopene synthesis includes four desaturation steps and need three enzymes Phytoene desaturase (crtP,Pds), zeta-carotene desaturase (CrtQ,Zds)
Cis-carotene isomerise (CrtH,CrtISO) [52][53][54]. First two desaturation steps are catalysed by CrtP, through phytofluene from phytoene to zeta-carotene. Two additional desaturation are catalysed by CrtQ with the help of neurosporene from zeta-carotene to lycopene. Lycopene can synthesise either alpha carotene or beta carotene through gamma carotene Astaxanthin synthesis Beta carotene is catalysed by CrtO to produce echineone and the product form finally is canthaxanthin . Crt W induces keto group in zeaxanthin , beta carotene and myxol which finally yield astaxanthin and 4-ketomyxol.[51][52][54]

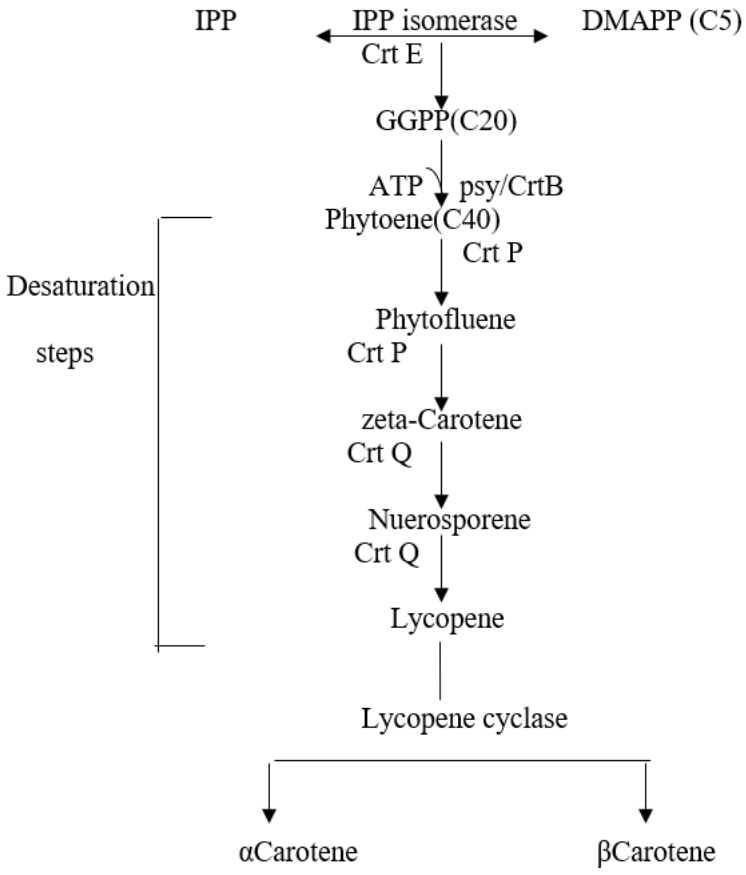


Fig2.2 - Pathway for the synthesis of beta carotene

2.6 SOLVENT BASED EXTRACTION OF PHYTOCOMPOUNDS

The solvent-based extraction depends on many factors. Some of the factors are discussed below

1. Polarity

Polar substance dissolves in polar solvent as “like dissoves like”. The choice of solvents depends on the nature of phytochemicals. It is important to study the relation between the solvent and properties of the compound to be extracted

2. pH

The pH of solvents is also one of the important factor during extraction of phytocompound. The non polar phytocompounds such as alkaloids can not be extracted in polar aqueous acid because of the alkaline nature and can result in formation of salt. The acidic phytocompounds are isolated with the help of solvent at basic pH.

3. Temperature

Generally the solubility of the mixture increases with the increase of temperature. The more the temperature, the more will be the penetration of solvents in the plant leaf (herbs). Furthermore , the thermo- labile compounds are sensitive to increased temperature. High temperature can degrade the bioactive compound and hence its properties, that can give rise to problems in extraction.

4. Nature of solvent

The solvents used can be volatile or non -volatile and polar or non polar . The phytocompounds have different extent of polarities. There the choice of solvent is an important factor. The basic features solvent are; non inflammable, easily removable, safe, no interactions, inert. Based on pH, a component can be an electrolyte or non-electrolyte that influence the solubility of solute and solvent.

Table 2.1 Different solvents used for extraction .

Nature of solvent	**Features**	**Examples**
Polar	Break covalent bonds of strong electrolytes. High dielectric constant. Solubility due to formation of H-bonds.	Water
Semipolar	These are intermediate solvents. Can induce miscibility of nonpolar and polar liquids	Acetone, Ethanol
Non -Polar	Solubility due to weak Vander Waals and London dispersion forces Low dielectric constant	Diethylether, chloroform , petroleum ether

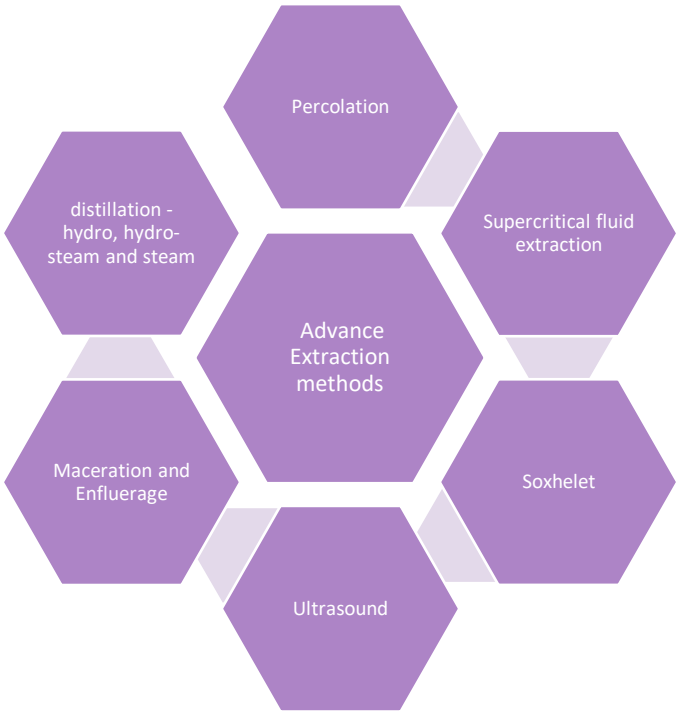


Fig 2.3 Different methods of phytocompound extraction from plants

2.8 UV-VIS Absorption Spectroscopy

The quantitative estimation of phytocompounds has been done using UV- VIS Spectrophotometer. It uses the Ultra -violet (UV) visible radiations.

Beer-Lambert law -"It states that, at a particular wavelength, absorption is directly proportional to the concentration of absorbing molecules and the pathlength of light passing through the sample"[55]

$$A = Ecl$$

where, A is absorption

E is molar extinction coefficient

L is pathlength

2.9 Future prospects

Phytotherapy have gained a lot of importance in nutraceuticals and as a alternate of medicine. The proper knowledge of bioactive compounds present in plant species and its effect on human race is of great importance. Proper identification of plant and its harvesting is a point to considerate. The activities of secondary metabolite is greatly influenced by the environmental factors and genetics of plant. The optimization of the nutraceutical compound is also a major challenge. Hence it must be propagated through biotechnological ways of tissue culturing. The production of plant species in controlled environment allows to study the pathway of phytocompounds of a particular plant species. The phytochemical analysis of metabolites is important for determining therapeutic activities.

CHAPTER 3. METHODOLOGY

3.1 Shade dried leaves

3.1.1 Preparation of aqueous plant extract

The leaves of 5 plants were collected from the Delhi Technological University campus. They were dried under shade for 5 days. 5 grams of Leaves were weighed and crushed with the of mortar pestle. The flask was taken, and 1 g of powder is added in 100 ml of water and are boiled. Once boiled leave the flask undisturbed covering with the aluminium foil. Left undisturbed until it cools down to room temperature. Centrifuge the solution at 4000 rpm for 10 minutes. Store the supernatant for qualitative test.

3.1.2 Qualitative estimation of flavonoids from shade dried leaves

Preparation of stock solution 1 – Acetone (1ml) + 10% aqueous potassium dichromate+ 6M sulphuric acid. The presence of blue green color is an indicative of presence of flavonoids in the plant. Added the 2ml of extract in the 1ml of stock solution and observe the blue green colour.

Preparation of stock solution of 1M NaOH +1M sulfuric acid .

5 ml of plant extract was taken and 1 ml of 1M NaOH was added and sulfuric acid dropwise. First it shows an intense yellow color and after adding the sulfuric acid if color disappears, it will indicate the presence of flavonoids in the sample.

3.1.3 Quantitative estimation of flavonoids

0.5 ml of plant extract added to 0.5ml Aluminium chloride prepared in 2% ethanol. The resultant mixture was incubated at room temperature for 60 minutes. This result in the development of yellow color that represents the presence of flavonoids in the sample. The absorbance was measured at 420 nm using UV-Vis Spectrophotometer.

Total flavonoid content was calculated using Quercetin as a standard. The standard dilution was prepared to find the concentration of unknown sample. Drawn a calibration curve where X axis represent concentration and y axis is absorbance.

3.1.4 Qualitative estimation of steroids from shade dried leaves

Preparation of stock solution – Add 10ml chloroform and 1ml sulfuric acid in the flask

1ml of plants extract when added to the stock solution. The red ring at bottom will indicate the presence of steroids.

3.2 SUNDRIED LEAVES

3.2.1 Preparation of plant extract

The leaves are collected from the Delhi Technological University campus. They are dried for 2 days under sunlight. Five grams of Leaves were weighed and crushed with the of mortar pestle. Now, took the flask and 1 g of powder is added in 100 ml of water and are boiled. Once boiled leave the flask undisturbed covering with the aluminium foil. Left undisturbed until it cools down to room temperature. Centrifuge the solution at 4000 rpm for 10 minutes. Store the supernatant for qualitative test. Performed the qualitative test for steroids flavonoids.

3.3 FRESH LEAVES

3.3.1 Preparation of plant extract -

The leaves are collected from the Delhi Technological University campus. Plant were washed in tap water. 5 grams of Leaves were weighed and crushed with the help of grinder. Now took the flask and 1 g of powder is added in 100 ml of water and are boiled. Once boiled leave the flask undisturbed covering with the aluminium foil. Left undisturbed until it cools down to room temperature. Centrifuge the solution at 4000 rpm for 10 minutes. Store the supernatant.

3.4 ORGANIC SOLVENT-BASED EXTRACTION

3.4.1 Preparation of methanolic plant extract

0.5 gram of the leaves dried powders of all the plants were weighed and added in 50 ml methanol separately. The flask were kept in B.O.D incubator at 40 degree Celsius for 48 hours. The flasks were taken out of the incubator and left undisturbed until it reaches to room temperature. Transferred the solution in the falcon tubes and centrifuge it at 4000 rpm for 10 minutes. Now filter the extract using Whatmann filter paper and funnel and supernatant were collected.

3.4.2 Preparation of plant extract using Petroleum ether

0.2 gram of the leaves dried powders of all the plants were weighed and added in 15 ml petroleum ether separately. The flask were kept in B.O.D incubator at 40 degree Celsius for 48 hours. The flasks were taken out of the incubator and left undisturbed until it reaches to room temperature. Transferred the solution in the falcon tubes and centrifuge it at 4000 rpm for 10 minutes. Now filter the extract using Whatmann filter paper and funnel and supernatant were collected.

3.4.3 Preparation of plant extract using Chloroform

0.5 gram of the leaves dried powders of all the plants were weighed and added in 50 ml chloroform separately. The flasks were kept in B.O.D incubator at 40 degree Celsius for 48 hours. The flasks were taken out of the incubator and left undisturbed until it reaches to room temperature. Transferred the solution in the falcon tubes and centrifuge it at 4000 rpm for 10 minutes. Then filtered the extract using Whatmann filter paper and supernatant were collected.

3.5 Extraction of β carotene from Red Carrot

Three red carrot was taken and were washed with distilled water. Peel off the carrot and cut it into small pieces followed by grinding. The grinded carrot was weighed and added in a separate container. 24 ml of Ethanol were added to the beaker along with 1ml of water. The container was covered with Aluminum foil. Now the container was kept at 40 degree Celsius inside the B.O.D incubator for 1 hour. Then the container was taken out from water bath and added 10ml of petroleum ether in the same funnel. Mix the solution properly. Take the supernatant add to cuvette. The absorbance was measured at 450nm

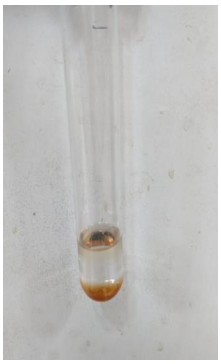
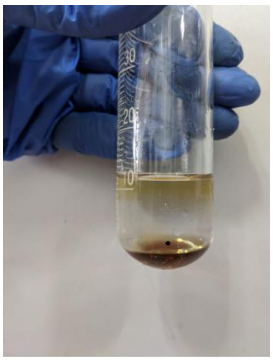
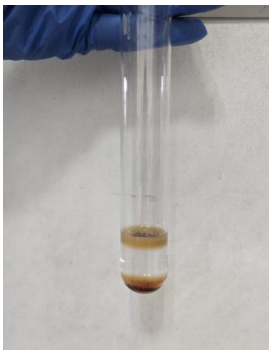
Standard dilutions were prepared using beta carotene as a standard.

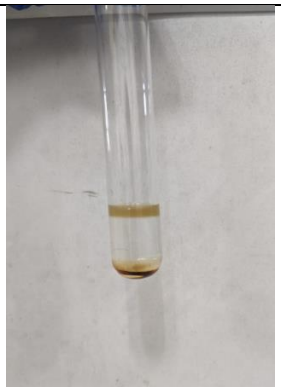
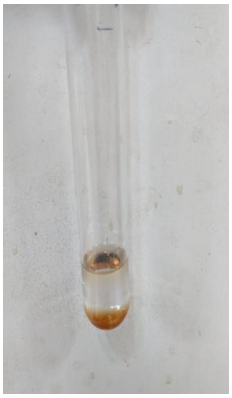
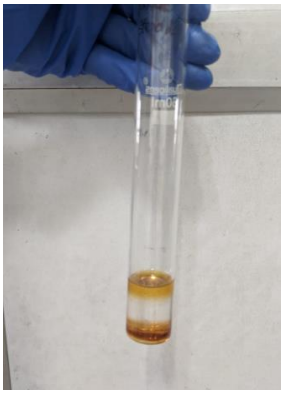
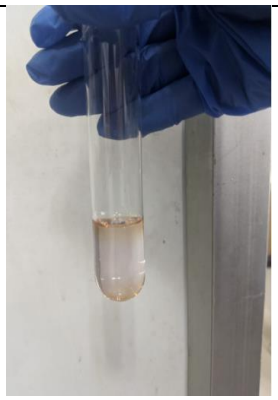
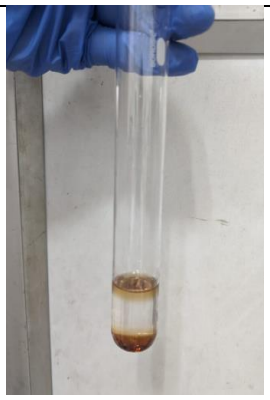
CHAPTER 4. RESULTS

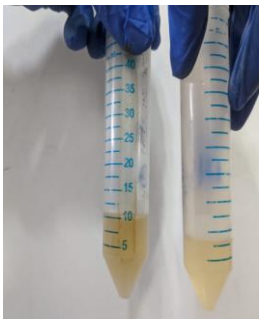
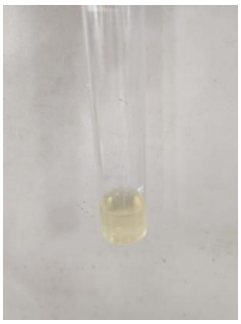
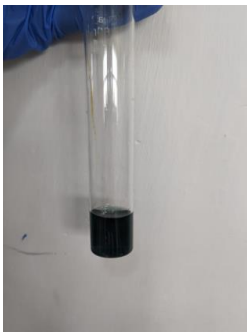
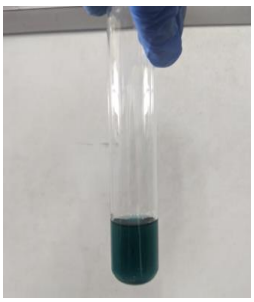
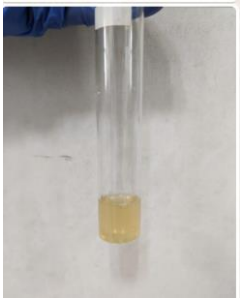
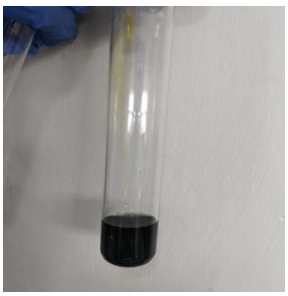
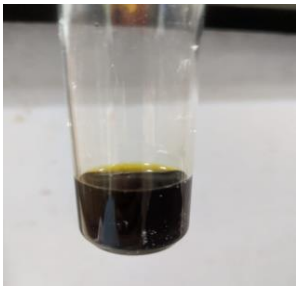
4.1 Qualitative estimation of Flavonoids

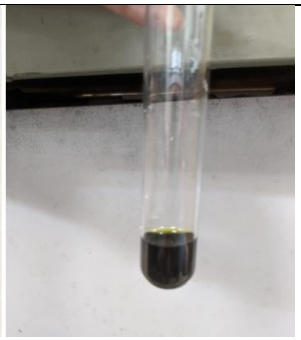
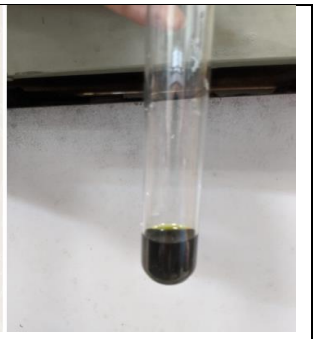
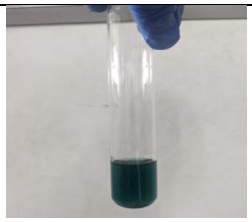
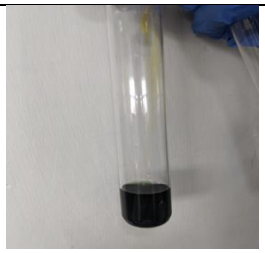
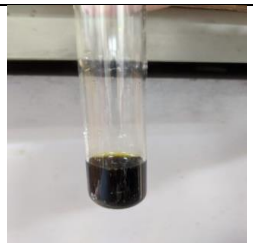
The indicative test for steroids was performed with all the plants collected from the nursery of Delhi Technological University and The results were observed as shown in the table 4.1.

Table 4.1 Qualitative analysis of steroids from sundried , shade dried and fresh leaves of different plants.

Plants	Sundried	Shade dried	Fresh
Moringa oleifera Brown ring at bottom. Positive test for phenol	 .		
Curry Brown ring at bottom. Positive test for phenol.			
Hibiscus			

Brown ring at bottom. Positive test for phenol			
Kalanchoe pinnata Brown ring at bottom. Positive test for phenol			
Catharanthus roseus	 Brown ring at bottom	 No brown ring observed , negative test for Steroids	 Brown ring observed

Plants			
	Shade dried	Sundried	Fresh
Moringa Oleifera			
Curry			 ++
Hibiscus rosasinsis	 ++	 ++	 ++
Kalanchoe pinnata			

	 ++		 --
Periwinkle			

4.2 Qualitative estimation of flavonoids

Table 4.2 Qualitative analysis of flavonoids from sundried, shade dried and fresh leaves of different plants.

4.3 Qualitative estimation of flavonoids in methanolic extract of plants

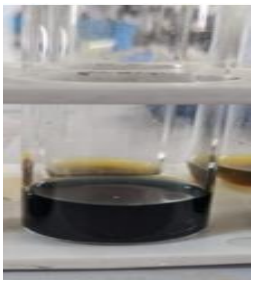
Moringa oleifera  A)	Curry  B)	Hibiscus  C)
Hibiscus rosasinesis  D)	*Kalanchoe Pinnata*  E)	

Fig.4.1 No change in color observed , all plants have shown negative test for flavonoids

4.4 Qualitative estimation of flavonoids in methanolic extract of plants

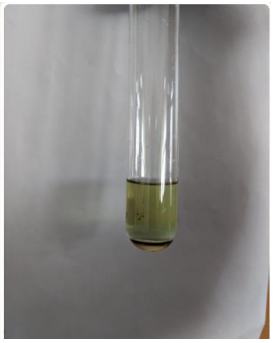
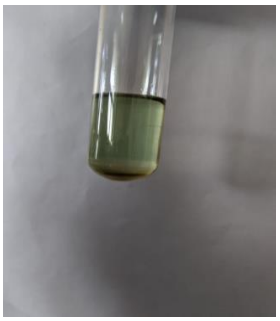
Moringa oleifera  A)	Curry  B)	*Catharanthus roseus*  C)
Hibiscus  D)	*Kalanchoe pinnata*  E)	

Fig. 4.2 All the plants have shown positive test for steroid

4.5 STANDARD CURVE FOR β-CAROTENE

Table 4.3 Absorbance at 450 nm of different dilutions using Hexane as a solvent

Dilutions (mg/ml)	Beta carotene (mg)	Hexane	Absorbance at 450
0.5mg/ml	3	6ml	3.1692
0.25	1.5	3ml	3.1017
0.125	0.5	2ml	3.0425
0.062	0.18	1.5ml	1.8208
0.031	0.06	1ml	0.9824

Table 4.4 The absorbance of standard dilution using Petroleum ether as a solvent

Dilutions (mg/ml)	**β carotene (mg)**	**Pet ether**	**Absorbance at 450**
0.5mg/ml	3	6ml	3.348
0.25	1.5	3ml	3.2508
0.125	0.5	2ml	2.3261
0.062	0.18	1.5ml	1.8487
0.031	0.06	1ml	0.992

Table 4.5 Absorbance of sample at 450 nm using hexane as a solvent

Amount of Sample (red carrot)	**Hexane (ml)**	**Sample extract**	**A450**
15g	10	10	0.1852
40g	10	10	0.2305
30g	10	10	0.0638

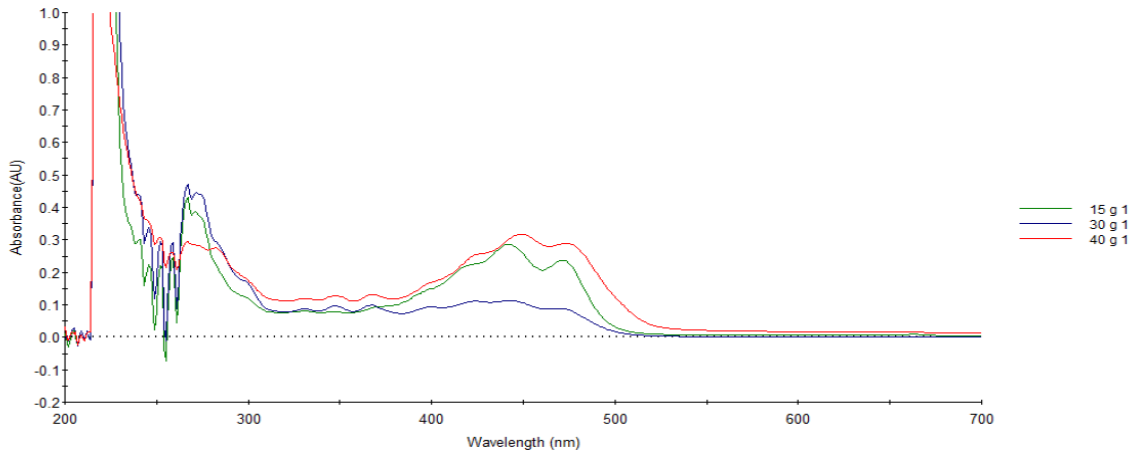


Fig. 4.3 Scan graph for βcarotene from red carrot obtained from UV- Vis Spectroscopy

Table 4.6 Absorbance of sample at 450 nm using petroleum ether as a solvent

Amount of Sample (red carrot)	**Water (ml)**	**Ethanol(ml)**	**Extract (ml)**	**Petroleum ether(ml)**	**A450**
15g	1.5	36	10	10	0.1852
40g	4	96	10	10	0.2305
30g	3	72	10	10	0.0638

Table 4.7 Amount of β-carotene present in the unknown samples

Amount of Sample (red carrot)	Water (ml)	Ethanol(ml)	Extract (ml)	Pet ether(ml)	A450	Amount of βcarotene obtained
15g	1.5	36	10	10	0.1852	0.2g/ml
40g	4	96	10	10	0.2305	0.25g/ml
30g	3	72	10	10	0.0638	0.1g/ml

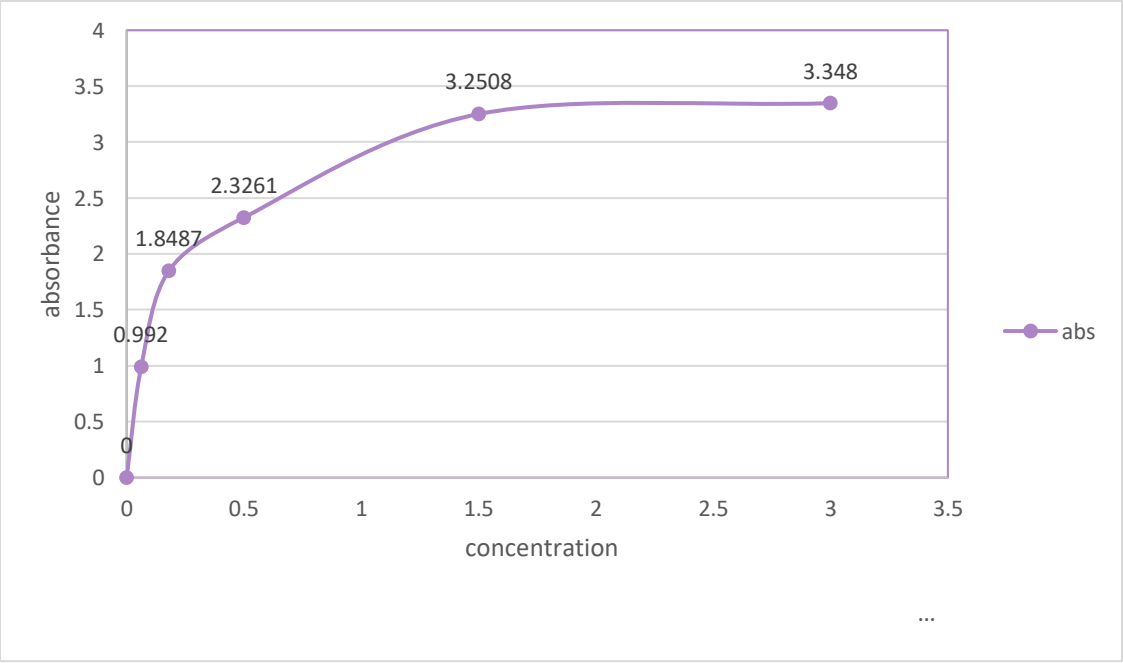


Fig 4.1 Standard curve obtained for β carotene.

Table 4.8 Standard dilution for flavonoids using Quercitin as standard.

Quercitin dilutions (mg/ml)	Aluminium chloride (ml)	A420
0.015	0.5	0.4203
0.03125	0.5	0.7857
0.0625	0.5	1.4006
0.125	0.5	2.1552
0.25	0.5	3.0588
0.5	0.5	4.69
1	0.5	4.7

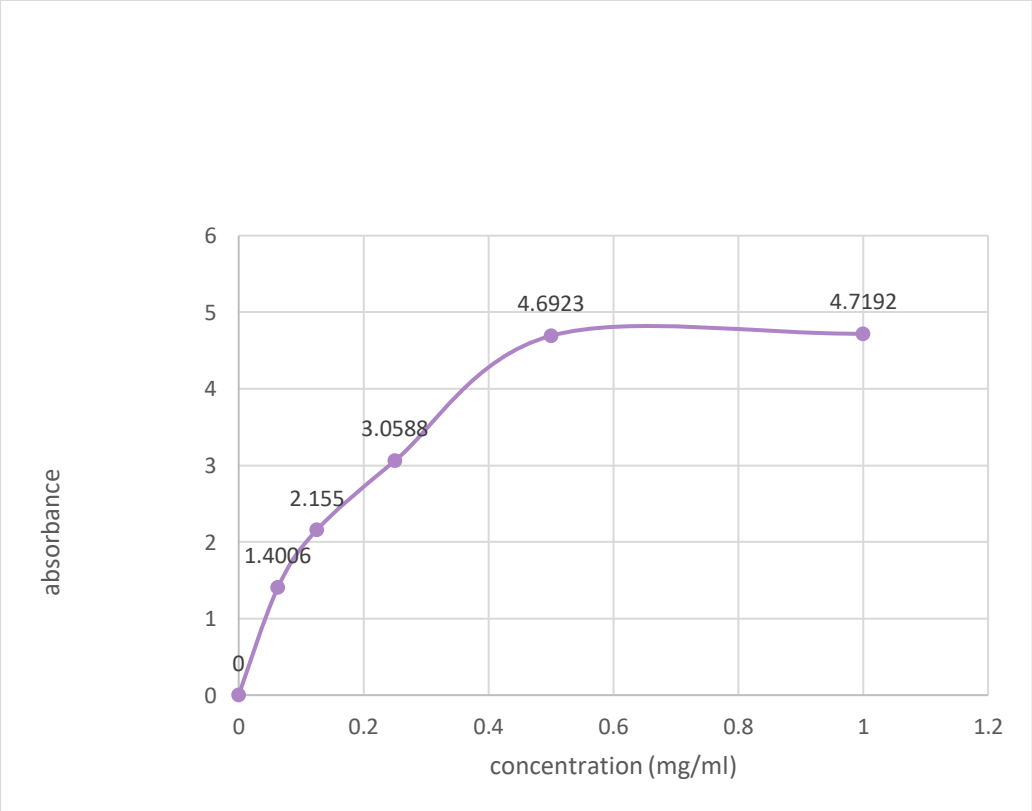


Fig.4.2 Standard curve for Flavonoids

Table 4.9 Amount of Flavanoids obtained in different plants

Sample	Solvent extract(ml)	Aluminium chloride	A420	Amount flavonoids
Moringa	0.5	0.5	0.5206	0.03mg/ml
Hibiscus	0.5	0.5	0.3152	0.01mg/ml
Periwinkle	0.5	0.5	0.3314	0.01mg/ml
Curry	0.5	0.5	0.7849	0.04mg/ml

Discussion

Secondary metabolites are very necessary for both humans and plants. The plant produces metabolites during its metabolism in natural conditions. Algae and Plants are a major source of nutraceuticals that are safe and is need of an hour. According to WHO, around 70% of the population is dependent on herbal medicines for treating the disease at the primary level. Therefore, now it becomes very important to search for more plants and algae species that have nutraceutical properties. The qualitative and quantitative estimation of plant bioactive compounds is one of the approaches to checking the presence and amount of secondary metabolites. The test was done after putting plant leaves under different conditions such as shade drying and sun drying, and the results were compared. The different solvent extraction method was performed. In the shade dried leaves, sundried and fresh leaves extract prepared in water all the test were positive for steroids and flavonoids. On the other hand the plant extract when prepared in organic solvents like methanol and pet ether . In case of methanolic extract the test for steroids were positive but for flavonoids the test was negative. In extraction with petroleum ether, all the test was negative both for flavonoids and steroids. Furthermore in case of Quantitative estimation of flavonoids from different plants . the standard curve was obtained using Quercitin as a standard. The amount of flavonoids was determined in the different sample of plants by standard curve. Moreover in the beta carotene quantitative estimation in red carrot , the standard curve was obtain using beta carotene as an standard and the amount of beta carotene in the different carrot was determined. The determination of the valuable compounds is necessary for the production of nutraceuticals from plants as well algae. This also plays a role in determining the biological activities of that particular metabolite.

Conclusion

The results obtained after phytochemical estimation is important. Various secondary metabolites are used to treat human acute and chronic diseases. Different part of the plants may contain valuable compounds that can used in pharmaceuticals products for example leaves of curry, *Moringa oleifera* and flowers of Sunflower. Plants being used from decades in traditional medicine and for the treating diabetes, cancer, arthritis etc. All the metabolites discussed in the project have antioxidant and anti-inflammatory properties Today when world is more prone to disease due to their poor lifestyle and need a strategic plan to remain healthy as prevention is better than cure. Intake of nutraceuticals during coronavirus pandemic also helped people to boost their immunity and prevent them from novel virus infection. Novel infectious disease will continue to threat world therefore effective broad spectrum natural drugs highly required for example current COVID 19 outbreaks when vaccines were not available, clinical drug development is too costly, secondary metabolites can be considered as wonder molecule as a therapy.

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I hereby certify that the project dissertation title **"Estimation of β carotene and other secondary metabolites in important medicinal plants"** which is submitted by **Garima, 2K20/MSCBIO/07,** Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the of Master of Science, is a requirement for the award of the degree of Master of Science, is a record for the project work carried out by the student under my supervision. To the best of my knowledge, the above work has not been submitted in partor full for any Degree or Diploma to this University or elsewhere. I, further certify that the publication and indexing information given by the student is correct.

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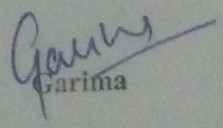

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