Screening of High triterpenoid and phenolic compounds containing population of *Ocimum* species and its ex-situ conservation

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Degree of

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In

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Under the Supervision of

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By

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Phagwara, Punjab (May 2015)

DECLARATION

I hereby declare that the project work entitled "Screening of High triterpenoids and Phenolics containing population of *Ocimum* species and its ex-situ conservation" is an authentic record of my own work carried out at Lovely Professional University as requirement of project work for the award of degree of Master of philosophy in botany, under the kind guidance of Dr. Gurdev Singh during the month of January to April, 2015.

IRSHAD AHAMAD BHAT

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I certified that the above statement made by the student is correct to the best of my knowledge and belief.

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CERTIFICATE

This is to certify that, IRSHAD AHAMAD is pursuing M. Phil dissertation titled "Screening of High triterpenoids and Phenolics containing population of *Ocimum* species and its ex-situ conservation.'' under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of this dissertation proposal/final report has ever been submitted for any degree or diploma. The dissertation proposal/final report is fit for the submission and the partial fulfilment of the conditions for the award of M.Phil in Botany**.**

DR. Gurdev Singh

Signature of Advisor

Department of biotechnology

Lovely Professional University

Phagwara, Punjab

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First of all ,I am grateful to Omnipresent ,Omniscient, &Omnipotent ,The Almighty , ALLAHA without whose grace I could never have done the work& to whom I am accountable for my every act done in this mundane world.

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Abbreviation

2, 4 D – Dichlorophenoxy acetic acid

BAP- Benzyl amino purine

IAA- Indole acetic acid

IBA- Indole Butyric acid

NAA- Napthalene Acetic acid

MS Media- Murashige & Skoog media

TLC- Thin Layer chromatography

HPLC- High Performance Liquid Chromatography

HPTLC-High Performance Thin Layer Chromatography

OA-Oleanolic Acid

UA- Ursolic Acid

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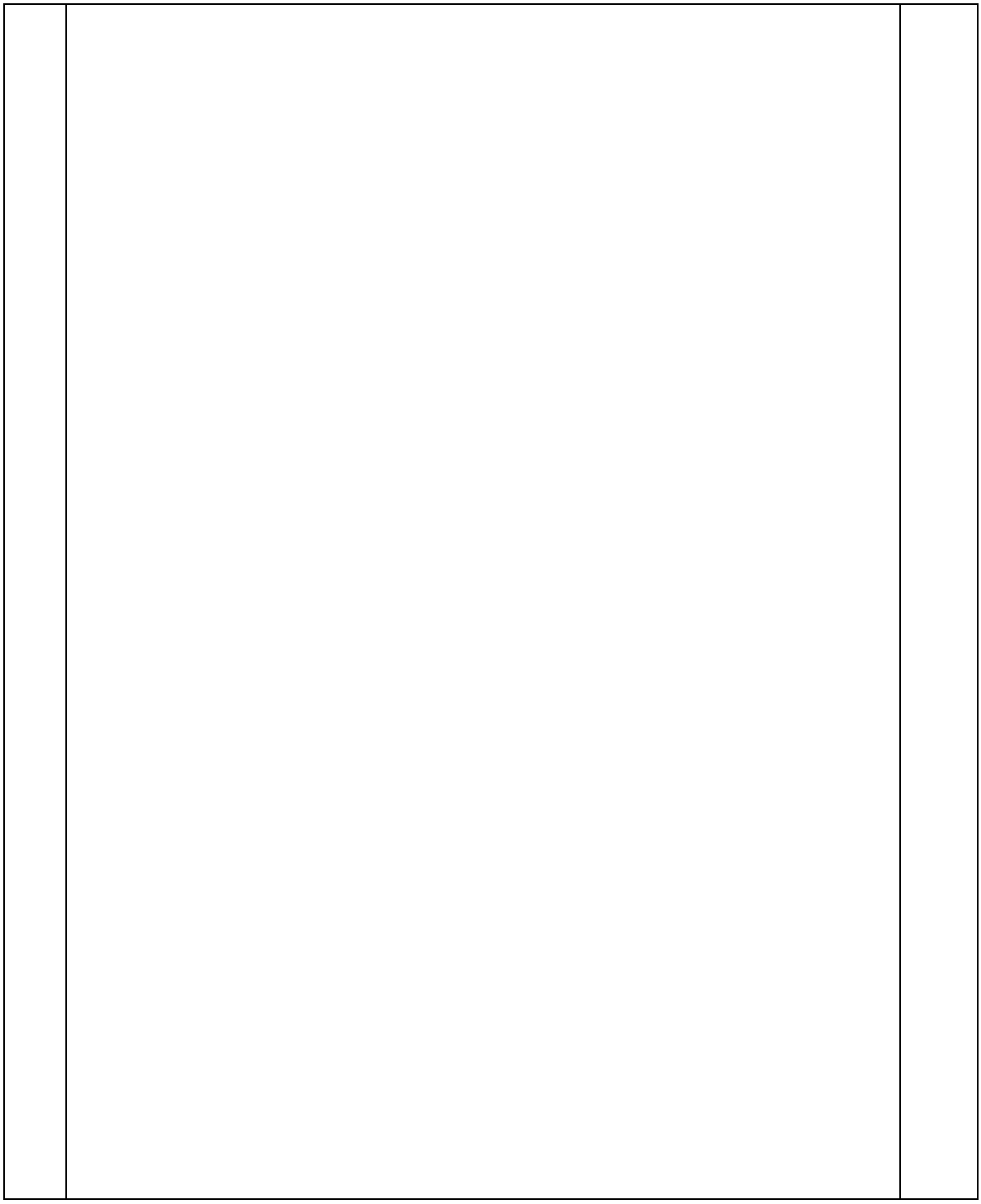

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Chapter 1

Introduction

Nature has bestowed on us a very rich botanical wealth .Since this is well known fact that plants are rich sources of drugs of traditional system of medicine, modern medicine, food supplements, pharmaceuticals intermediates, and chemical entities for synthetic drugs **(Hammer *et., al* 1999)**.Medicinal plants have generated a great economical value all over the world. Today majority of drugs in market are obtained from plants, like Morphine from *Papaver somniferum*, Aswagandha from *Withania somniferum*, Ephedrine from *Epherda Vulgaris*, Atropine from *Atropa belladonna*, Reserpine from *Roulphia serpentine* etc. Medicinal plants are potent source of Secondary metabolites (sources of drugs) and essential oil of therapeutic importance. The important advantage of these medicinal plants lies in the fact that the drugs obtained are safe as well as economical, cost effective and their easy availability **(Kapoor *et al.,* 1990)**. According to a survey (2005) of World Health Organisation, the practitioners of traditional system of medicine treat about 80% of patients in India, Burma and 90% in Bangladesh **(Siddiqui HH *et al.,* 1993)**.

Ocimum species (Lamiaceae), in general known as 'Basil' are annual or perennial, highly aromatic, branched herb or shrub native to tropical and subtropical regions of Asia **(Labra *et al*.,2004)**.The genus *Ocimum* comprises nearly about 150 species and is regarded as one of the biggest genus of family Lamiaceae **(Pushpangadan and Bradu,1995)**.The main representatives of this family which are known to possess medicinal properties includes *Ocimum sanctum, Ocimum basilicum, Ocimum gratissimum,* and *Ocimum canum* . There has been a tremendous growth in the world business for essential oils (growing nearly to 11% per year **(Bizzo *et al.,* 2009)**.The trade and internal consumption of these oils have shown considerable increase in India also in recent past. In Indian these oils are produced from different species and those belonging to the genus *Ocimum* contribute to the total exports.

Ocimum sanctum generally known as 'tulsi' in Hindi is a 30-75 cm erect herb which is grown in every part of India. Leaves of this plant are 2-2.5cm long and 1.6 -3.2 cm broad, elliptical. The utilisation of this very herb has been reported in Indian traditional System of Medicines and its modern applications are receiving broad spread attention day by day. It has been observed in different research works to possess antioxidant, antibiotic, immunomodulatory, anti-inflammatory, analgesic, antiulcer and antipyretic properties **(Singh**

***et al*., 2007)**. *Ocimum gratissimum* occurs all over India and is cultivated for its essential oils which have been known to possess antimicrobial activity **(Ananda et al., 2010)**.*Ocimum basilicum* is very rectified spice and is grown condimental plant as well in Turkey **(Ozcan *et al*., 2002)**.Scientific studies have confirmed that bioactive compound present in the sweet basil have potent antioxidant activity , it has been also proved that it has anti-cancerous ,anti-viral, anti-aging **(Bozin, *et al*., 2006)**.Because of the anti-oxidative property, basil has been used to enhance the shelf life of food stuffs.

Since in current the researchers are putting more efforts to find the natural sources of antioxidants as now people are more aware about their diet and the synthetic antioxidants use have been limited now-a-days due to their carcinogenicity .So there need to find the natural sources of antioxidants. Spices are known to possess good content of antioxidants and some of spices even exceed the synthetic antioxidants, and are safe to use because of natural origin. Among that basil has much importance due to its versatile uses as herb as well as spice.

Standardisation of drugs gives hint towards the identity and determination of its quality and pureness. Now-a-days due to progress in the chemical knowledge of crude drugs various methods like botanical , chemical, spectroscopic and biological methods are used for estimating active constuients present in the crude drugs in addition to its physical constants. The process of extraction for bioactive ingredients in plants for a researcher has always been a challenging task .With the increasing demand for health care approaches, revitalization of herbal drugs have played tremendous role in changing the health care situation across the world .But diagnosis of elite variety is of utmost importance to deliver quality products to the global market. Hence, the need of hour is to employ the sophisticated techniques towards the standardisation of single herbal preparations .The evaluation of a product is its entirety , so called “fingerprinting” can be accomplished by appropriate methods ,which may include HPLC, GC/MS,HPTLC-densitometry, FT-NIR, high-field NMR or a combination of these techniques. Chromatographic fingerprinting is highly informative which includes its use as an absolute indicator of the chemical characteristic of plants. Adulterants can be distinguished even in processes samples, enabling the authentication of the drug. the fingerprinting of chemical profile as well as the quantification using analytical techniques are comprehensive and the different varieties of *Ocimum* species exemplified that fingerprinting using analytical technique are comprehensive and more information to identify and authenticate the raw drugs and proves to be tool for standardisation of herbal drugs

Chapter 2

Review of literature

2.1 Holy Basil: The Elixir of Life

One of the most scared herb of India as well as an intrinsic part of ancient Hindu traditions. According to Hindu scholars, the origination of *Ocimum sanctum* has been considered one of the 14 "Ratans" from ocean as the proximate scared herb to cure diseases. From the Hindu mythology point of view Holy basil is believed to be the reflection of the Goddess Lakshmi, the wife of Vishnu, and is worshipped for health and wealth **(Raina *et al*., 2013)** .Holy basil is well- advised as one of the rectified blessings from the God and has been described as a magic herb in many ancient medicinal texts such as Siddha, Ayurveda, and Unani **(Engles and Brinckmanm, 2013)**. Keeping all these things in mind Holy basil has been grown permanently in Hindu homes, temples, and other important places. Leaves are generally the most important part having medicinal property like anticancer, antistress, antimicrobial and antipyretic **(Engles and Brinckmann, 2013)**. Holy basil has been considered as "Queen of Herbs".

Botanical description of the plants

Ocimum sanctum has been placed in Family Lamiaceae, and is found throughout tropical and semitropical region of India and other Asian countries **(Godhwani *et al,* 1988; Pattanayak *et al*., 2010)**. It is a branched and erect herb .Plant attains height of about 75 to 90 cm. Leaves are almost round and up to 4 cm long with margin, i.e. entire or toothed. Flowers are purple to reddish colour, having compact cluster or cylindrical spike. The fruits are small in size .The fruits may be yellow to reddish in colour. Traditionally the plant parts use is mentioned in the Ayurveda and Siddha systems. The treatment according to these include several aliments like infection, skin disease, hepatic disorder, common cold and cough, malarial fever and as an antidote for snake bite and scorpion sting **(Godhwani *et al,* 1988).**

Scientific classification **(Pattanayak *et al*., 2010)**

Kingdom	Plantae
(unranked)	Angiosperms
(unranked)	Eudicots
(unranked)	Asterids
Order	Lamiales
Family	Lamiaceae
Genus	*Ocimum*
Species	*O. sanctum*

Ocimum gratissimum is an aromatic, perennial herb, 1-3 m tall; stem erect, round, much branched, woody at the base. Leaves opposite; petiole 2-4.5 cm long, slender, pubescent; blade elliptical to ovate, 1.5-16 cm x 1-8.5 cm, sometimes glandular punctate, base entire, margin elsewhere coarsely serrate. Inflorescence a verticillaster arranged in a terminal, simple or branched raceme 5-30 cm long; flowers in 6-10-flowered verticillasters, small, hermaphrodite, 2-3 mm long. Fruit consisting of 4, dry, 1-seeded nut lets enclosed in the persistent calyx

Kingdom	Plantae
(unranked)	Angiosperms
(unranked)	Eudicots
Order	Lamiales
Family	Lamiaceae
Genus	*Ocimum*
species	*O. gratissimum*

2.2 Pharmacological potential of *Ocimum sanctum*

2.2.1 Antioxidant activity.

O. sanctum antioxidant potential has been observed by various researchers **(Madhuri S., Pandey Govind.Biomed 2010)**. Antioxidant activity of the flavonids (orientin and vicenin) in vivo was stated in an important decrease in the radiations prompted lipid peroxidation in mouse liver **(Uma Devi p.Indian.2001)**

The phenolic compounds, viz., rosmarinic acid and eugenol (the main constuients present in the essential oil of *Ocimum sanctum*) possess remarkable antioxidant property **(Nair AGR *et al*., 1982).**

2.2.2 Immunomodulatory effect.

The most important pharmacological role of Tulsi is the enhancement of immune response by increasing the cellular and humoral response. This is achieved by acting at various points in the immune mechanism such as release of hypersensitivity mediators, assembly of antibodies and reply to thes mediators on the target organs. **(Mediratta PK, Gupta VS, 1998)**.

2.2.3 Antipyretic activity.

Ocimum sanctum oil was found to have antipyretic activity. The antipyretic potential of oil was assessed by checking it against typhoid-paratyphoid A/B vaccine-induced pyrexia in rats. During the IP administration of oil, it clearly showed the antipyretic action. At a dose of 3 ml/kg, the antipyretic activity of the oil was similar to aspirin. **(Singh S, taneja M Majumdar DK, 2007.).**

2.2.4 as an anticancer agent.

Cancer has been a foremost cause of death. With changing standard of living and food habits. Cancer is outstanding all other ailments as a principle cause of death .It has been found that the ethanolic extract of *O.sanctum* intermediated a significant in tumour cell size and an enhancement in of mice life span having cancer **(BabyJoseph and Vrundha. m .N ,2013)**

2.2.5 Analgesic activity.

Analgesic activity of *OS* extracts was found to be almost negligible in experimental plain models (tail flick, tail clip and tail immersion methods). However, it was observed that *OS* extracts were effective against acetic acid induced writhing method in mice in a dose dependent manner. **(Singh S, taneja M Majumdar DK, 2007.)**

Pharmacological potential of *Ocimum gratissimum*

Pharmacological potential of *Ocimum gratissimum* is believed to be due to the different phytochemical constuients present in the plants that ultimately lead to many positive effects on human body. Lot of medicinal benefits of *Ocimum gratissimum* can be seen in medical journals mostly from studies using in vitro bioassays and small clinical trials. However, some of the medicinal properties include: antifungal activity, results revealed that essential oils inhibited the growth of *Rhizoctonia sp*. and *Alternaria sp*. **(Terezinha JF et al., 2006),** antibacterial of steam distillation extract have shown inhibitory effects against *S. typhimurium* and *S . typhi* **(Adebolu TT *et al*., 2005)** , ovicidal activity was evaluated against *Haemonchus Contortus* showed that essential oil from *Ocimum gratissimum* can be utilised to control gastrointestinal helmintosis **(Pessoa LM *et al* .,2002)**, analgesic activity was tested in rabbit and mice and the results positive confirmation, nematicidal activity was found to be by aid of Eugenol **(Chatterje *et al* ., 2005)** , antioxidant activity of methanolic extract had DPPH scavenging activity of 84.6% at 250µg/ml, Antidiabetic effect was tested in streptozocin-induced diabetic rats and the results confirmed that *O. gratissimum* possess Antidiabetic activity in streptozocin-induced diabetic rats **(Akinmoladun AC *et al*.,2004)** , cardiovascular effect was investigated in rats by intravenous administration of essential oil and results suggest that hypotensive activity of EOOG results in vasodilation **(Lahlou s *et al*.,2004)**, anti-inflammatory effect of essential oils from *O. gratissimum* was found to inhibit cyclooxygenase enzyme involved in production of inflammation mediators **(Obaseiki- Ebor et al.,2002)**

2.3 Phytochemical constuients of TULSI: the elixir of life

Large number of technologies have been utilised for quantification of the phytochemical present in the medicinally important plants including gas chromatography (GC), high performance thin layer chromatography (HPTLC) , GC- mass spectrophotometry (GC-MS), 1D and 2D NMR Spectroscopic analysis, Solid phase microextraction (SPME), flame ionization detection (FID), and olfactoric evaluation **(Amber et al., 2010)**

The unique aromatic odour of *O. sanctum* is because of the essential or volatile oils. The aromatic volatile oil mainly constitutes phenols, terpene and aldehydes. Various studies proved that chemical ingredients vary due to edaphic and geographic factors **(Bakkali F, Kumar. 2008)**

Besides oil, the plant also contains alkaloids, saponins and tannins. The volatile oils are mainly concentrated in the leaf. Ariel parts of *O. sanctum* possess 0.7% volatile oil comprising of two major components eugenol and methyl eugenol **(Patil KS, Bhardwaj LK., *et al* 2010)**

The essential oil from leaves encompass α- Thujene, Ethyl benzene, Cis- β - ocimene, Trans- β - ocimene, terpene, Geraniol, linalool, eugenol, methyl eugenol, Isocaryophyllene, β – caryophyllene and iso- eugenol **(Philip MP ,D amodaran 2007)**

The alcoholic extract of leaf and other aerial parts possess ursolic acid, stigmsterol, chlorgrnic acid, gallic acid, gallic acid methyl ester, gallic acid ethyl ester, vallinin, caffiec acid, chlorogenic acid, β- stigmsterol and rosmarinic acid **(Sukrari MA, Rahmani, et *al* .1995)**

Differences in chemical profiles were observed from oils possessed by different species from the same population and location of *Ocimum* indicating the presence of different chemotypes within this species. The chemical composition of different varieties varies and is dependent upon the geographical location and cultivars. *O. basilicum*, showed clear variation in phenotype and chemotype in terms of oil content and its composition **(Zhelijazkov *et al*., 2008)**.

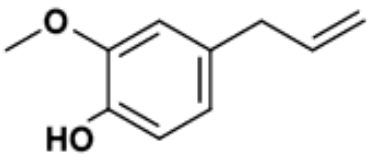
The qualitative tests was performed on the extracts obtained from the ariel plant parts of *Ocimum sanctum* revealed the presence of phytochemical ingredients such as tannins, cardiac glycerides, flavonids, tannins, Terpenoids, and saponins . It has also been confirmed

that the phytochemical constituent like phlobatannin were lacking .The major constituents found in *Ocimum sanctum* was Eugenol (43.88%), caryophyllene (26.53%). **(G. Devendran and U. Balasubramanian., 2011)**

Important pharmacological compounds in *ocimum sanctum* and *gratissimum*

Eugenol

Eugenol belongs to a class of chemical compounds known as phenylpropanoids .Eugenol is a pale yellow liquid obtained from certain essential oils particularly from clove oil, basil and cinnamon. Food industry use eugenol as a flavouring agent, has a numerous biological activity, and can be used as biomarker. Eugenol is the leading constuients found in essential oil of *Ocimum* and has been widely recognised to possess analgesic action in dentistry. Eugenol is known to possess pharmacological effects on almost all systems. Beside this eugenol is known to possess other pharmacological effects like antioxidant, antiinflammatory and cardiovascular properties. Keeping all these points in view eugenol seems to be very promising candidate for the versatile application and the designing of new drugs based on the medicinal effects of eugenol.

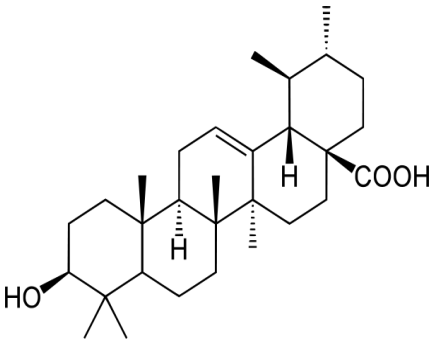


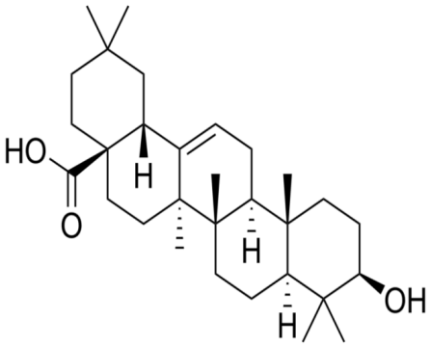
Eugenol

Oleanolic and ursolic acid.

Oleanolic and ursolic acid are rectified triterpenoids compounds that are found in medicinal herbs and other related plants .These triterpenoid possess lot of medicinal properties. Both of these compounds are known to provide liver protection against chemically induced liver

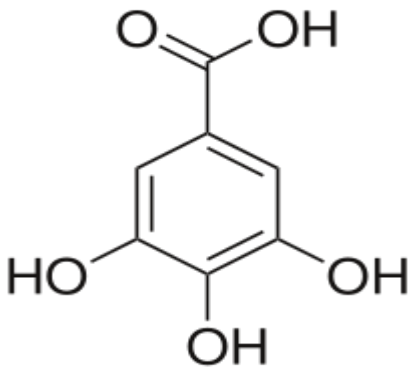
injury in laboratory animals. China has given recommendation on oleanolic acid to be marketed as an oral drug for human liver disorders. The mechanisms by aid of which these two triterpenoids provide hepatoprotection involve inhibition of toxicants activation and enhancement of body defence mechanism. These two triterpenoids have also been known to possess antiinflammatory and anti-hyperlipidemic properties. Recent research has also confirmed that these two triterpenoids possess anti-tumour effects also. Oleanolic and ursolic acid are being used in cosmetics because they are non-toxic.

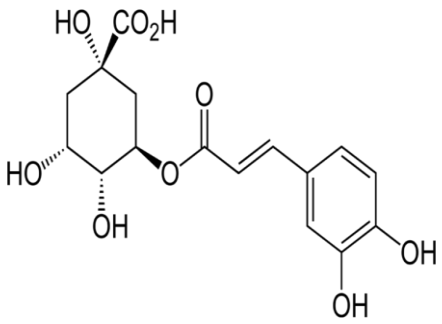




Structure of Oleanolic acid **Structure of Ursolic acid**

- **Gallic acid (**3,4,5- trihydrobezoic acid) is a naturally occurring plant phenol obtained by the hydrolysis of tanins and show some pharmacological activities. Gallic acid was found to have cytotoxicity against primary cultured rat hepatocytes and macrophages, and lesser cytotoxixity against fibroblast and endothelila **(fiuza,2004)**.





- **Structure of gallic acid** **Structure of chlorogenic acid**

2.4 Analytical methods

Leaf extracts of *Ocimum sanctum* were subjected to GC-MS analysis which revealed the presence of phytochemical constuients such as tannins, saponins, steroids, phenolics, flavonids, terpenoids and cardiac glycerides. The main bioactive compounds confirmed in the leaves of *Ocimum sanctum* are Eugenol (43.88%), caryophyllene (26.53%), gallic acid, chlorogenic acid, ursolic acid, cyclohexane and cyclopentane. The analysis revealed that there are mainly two bioactive compounds present in leaves i-e Eugenol and caryophyllene **(G.Devendran and U. Balasubramanian 2011)**

Different methods were employed for extraction of essential oils from *Ocimum basilicum* like (maceration, sonication and extraction in microwave).The extract were further subjected to TLC/HPTLC technique which confirmed the presence of eugenol, limonene, geranoil, gallic acid, citronellol, linalool and some terpenoids. Further the most efficient extraction technique was maceration followed by microwave .The feasible extraction solvent system was found to be ethyl ether+ ethanol (1:1,v/v).The TLC fingerprints of extracts obtained with different techniques showed to be similar**(Maria loredana *et al*., 2009)**

Ocimum sanctum plants were subjected to exposure of sUV-B, by aid of which there was increase in the oil content. The percentage content of essential oils was 0.86% in control and it increased to 1.22% in sUV-B exposed plants on the basis of fresh weight. In this research article GC/MS was utilised for the quantification of holy basil phytochemicals, which revealed that it contains mainly Eugenol ,ursolic, gallic acid, chlorogenic acid ,oleanolic acid and vicenin. **(Rima Kumari *et al*., 2010)**

The phenotypic features and essential oil content observed in different species of *Ocimum* was found to vary from 0.20 to 0.70%.The result from essential oil content clearly indicate that there is great variation between and within the species. These changes are due to fact of various intrinsic and extrinsic factors. Essential oils variation was confirmed by GC/MS**(Ram S. Verma *et al.*,2012)**

Quantification of phenolic compound present in aqueous extract of *Ocimum sanctum* by HPTLC was performed by utilising the mobile phase like toluene : ethyl acetate : formic acid (90:10:10).The retention factor of eugenol was found to be 0.58 . Furthermore the confirmation of phenolic compound (eugenol) in the sample extract was deep rooted by

overlaying the UV absorption spectrum of the sample with that of reference standard **(Nargis khan and sharique A Ali , 2014)**

GC/MS analytical technique was employed to check the variation of essential oil in *Ocimum sanctum* and *Ocimum basilicum*, in order to confirm the effect of the locality and seasonal variation on the constuients. The amount of oils ranged from 0.4 – 1.7% From this research work it was concluded *Ocimum sanctum* should be harvested in April for methyl chavicol **(Nargis khan and sharique A Ali , 2014)**

HPTLC method was developed according to ICH guidelines for identification and perfect quantification of ursolic acid in different parts of *Ocimum* species. Considerably excellent separation was obtained using toluene: accetone: formic acid (7.8;2.2:0.15, v/v/v) as a mobile phase. From the analysis it was clearly found that in *Ocimum* stem possess a good content of ursolic acid than leaves and flower.**(Kedar *et al.,* 2013)**

Ultra-violet spectroscopy and high performance liquid chromatography method have been developed for quantitative analysis of the bioactive compounds in the leaves of *Ocimum sanctum* and *Ocimum gratissimum*. From UV analysis, it was found that *Ocimum gratissimum* possess10.38 mg (0.20%) of Eugenol while as *Ocimum sanctum* has 13.91 mg (0.27%) of Eugenol. HPLC analysis revealed that the *Ocimum gratissimum* contains 8.28 mg (0.16%) of Eugenol while as *Ocimum sanctum* contains 10.08 mg (0.20%) of Eugenol. Finally, it was concluded from the results obtained from UV and HPLC that the *Ocimum sanctum* contains good content of eugenol.**(Sharma V 2011)**

2.5 Extraction methods

Hydro distillation- Water distillation was performed for the extraction of essential oils using an improved Clevenger trap .The time period for distillation was 1 hour for dried sample and 1 hour 15 minutes for fresh sample. Essential oil content was then estimated on the oil volume to tissue weight (fresh/dry) basis. Furthermore the volume of solvent utilised was 1000 ml for 75 g dry sample and 400 ml for 200 g fresh samples. **(Asta, 1968)**.

According to **(Massimo Labra *et al* ., 2004)** fresh leaves form *Ocimum sanctum* were taken for extraction purposes by utilising 150 ml of water in a 500 ml flask. This mixture was subjected to hydro- distillation until required amount was recovered. The collected distillate

was extracted with ethyl ether. Ethyl ether was removed at room temperature and essential oil was diluted with ethyl acetate

Steam distillation- Round bottomed flask containing dried or fresh plant material of *Ocimum sanctum* was utilised for carrying out steam distillation by passing steam into it for almost 90 minutes .After this time interval condensate (water/oil) was collected .The collected condensate was extracted 3- 4 times with solvent viz ethyl ether to extract essential oils completely. Sodium sulphate was added to remove moisture from condensate .Finally ethyl ether was removed by rotatory evaporator .Essential oil content was determined on weight (dry/fresh) basis. **(Burbott and Loomi, 1967)**.

Solvent extraction- solvent extraction of essential oil was conceded out by the method of **Burbott and Loomis (1967)**. Plant material was ground in a mortar containing hexane and anhydrous Na_2SO_4 and extracted four times with hexane to give a total volume of 10 ml of yellow extract. Silica vials were utilised for storage of essential oils.

Methanol extraction- Ariel parts of *Ocimum sanctum* were subjected for extraction purpose by taking required quantity in methanol for maceration. This methanolic extract was shaken frequently for first 6 hours and was then allowed to stand for 18 hours. Finally extract was subjected to filtration by using filter paper to remove impurities if present .The extracts were concentrated by utilising vacuum distillation to reduce the volume of methanol. **(Sharma V., 2011)**

Soxhlet extraction-According to the requirement plant samples for *Ocimum sanctum* were weighed accurately for extraction purpose in the solvent namely dichloromethane: methanol (1:1) in a Soxhlet extractor for 14 hours after initial defatting with n- hexane .Finally the extracts were concentrated and filtered through whatman No 1 filter paper. **(Kedar kumar *et al*., 2012)**

2.6 Micropropagation.

Due to recent advances in plant biotechnology tremendous ways have been provided by it for collection, multiplication and short to long term conservation of plant biodiversity. **(Carlos Alberto *et, al.* 2013)**

2.6.1 Micropropagation of "Holy Basil" from young inflorescences of mature plants

It has been performed by using MS media by talking explant as young inflorescence .Direct multiple shoot were differentiated by taking explant from inflorescence with 2- 3 weeks of time period after inoculation on MS media provided with BAP. The other observation revealed from this experiment was that when MS media was provided only with auxin, non-morphogenetic was produced callus. Furthermore it was also seen that incorporation of (IAA) along with (BAP) showed marked increase in the number of shoots **(Nirmal K. Singh *et al* ., 1996).**

2.6.2 Somatic embryo formation in *Ocimum sanctum*

MS media provided with 2, 4- Dichlorophenoxyacetic acid (2, 4-D) and benzyladenine (BA) was used for initiation of callus. The1 mg/l BA was found to be appropriate for the development of callus .Maximum variation into globular shaped somatic embryo was also detected at the same above mentioned concentration **(Rebecca Mathew *et al* 2011).**

2.6.3 Protocol Establishment for invitro-multiplication of *Ocimum sanctum*

Shoot tip and leaf explants of *Ocimum sanctum* were cultured on different concentration and mixture of growth regulators (BAP, Kin, 2,4-D, IAA and IBA) in MS medium to observe shoot multiplication .Callus induction was observed in MS media possessing 1.0 mg L^{-1} NAA. Shoot regeneration was attained at 0.2 mg L^{-1} **(L.A. Banu and M.A,Bari,2007)**

2.6.4 Callus – mediated plantlet regeneration of *Ocimum sanctum* using axillary buds as explant.

Callus established from axillary bud cultured in MS media provide with phytohormones viz. indole -3 acetic acid(IAA), α – naphthalene acetic acid (NAA) , 6-Benxyl aminopurine 9 (BAP) and Kinetin (Kn) either singly or in composition .Maximum number of shoots was

acquired using the concentration NAA as 1.3mg/l and 2.32mg/l Kn in combination. The developed shoots showed best rooting response when cultured at MS+26.85µM NAA+02.32µM Kn **(Kiran Gogoi *et., al* 2011).**

2.6.5 Protocol establishment for Multiplication and Regeneration of *Ocimum sanctum*.

Young shoot cultured on MS media containing hormones in different concentrations like IAA, NAA, BAP and 6% sucrose .The callus was further grown in fresh media after a definite interval of time. The maximum number of shoots was done with medium containing BAP. For root induction, MS media was provided different concentration of NAA (0.5–2.0 mg/l) Or IBA (0.5–2.0 mg/l).**(Begum M .N Amin *et al.*, 2010)**

Ocimum basilicum regeneration protocol was developed taking explant from leaf. Results suggested that Induction of callus was initiated on MS media supplemented with Thidiazuron (4 mg l^{-1}) .Calluses on medium containing Thidiazuron having more concentration turned brown and did not regenerate. Furthermore it was confirmed that when basil explants were placed onto medium having no growth regulators, number of adventitious roots developed within the time period of 7-10 days **(Winthrop *et al.*, 2000)**

An effective method has been developed for plant regeneration through somatic embryogenesis for *Ocimum basilicum.* Initiation of callus from leaf explants on MS media supplemented with 2, 4-D (1.0mg/l) and BAP (1.0mg/l), 3 % sucrose and 0.9% agar. The initiated callus showed clear differentiation into globular shaped structures, when transferred to MS media having lower concentration of auxin. **(C. Gopi and Ponmurugan, 2006)**

Rapid micro-propagating method has been developed by **(Y. Sahoo *et al.*, 1997)** for *Ocimum basilicum.* From nodal explants axillary shoot formation was induced on MS media (1962) provided with N-benzyladenine (BA) having 1.0 mg/l concentration on the first stage while as further growth and proliferation was done on MS media having a relatively low concentration of BA i-e 0.25 mg/l. By the aid of GA3 having concentration of 0.4mg/l axillary shoot formation was induced.

Chapter 3

Objectives

1) Collection of different species of *Ocimum* from the various geographical location of India.

2) Extraction and analysis of Terpenoids and phenolics from the sample extract of *Ocimum* species.

3) Quantative and Qualitative analysis of triterpenoids and phenolic compounds in plants collected from different geographical locations by spectrophotometry, HPLC and TLC respectively.

4) Screening of elite variety and its ex-situ conservation.

Chapter 4

Material and methods

4.1 Sample collection

Different *Ocimum* species were collected from two sates of India- Punjab and Himachal Pradesh .All samples were collected in vegetative state as well as in post flowering stage. The specimens were recognized and authenticated on the basis of morphological characters and by direct comparsion of herbarium specimens by Dr. D.K Pandey (COD Botany LPU, Phagwara Punjab). Plants used in the study were grown in earthen pots filled with soil, kept under shade and frequently watered.

Pictures (1) taken during collection of plant material *O. sanctum and O. gratissimum* respectively

4.2 Plant Sample extraction

Leaves and stem was cleaned, shade dried and pulverised to powder in mechanical grinder. Required quantities 5 g of powder was weighed and was extracted with dichloromethane – methanol (1:1) in a Soxhlet extractor for 30 hours after initial defatting with n-hexane.

Extracts were concentrated and filtered through Whatman NO.1 filter paper. **(Kedar *et al.*, 2012)**

Leaves were cleaned, shade dried and pulverised to powder in mortar and pestle. Required quantity of powder was weighed and transferred to Stoppard flask, and treated with hydroalcohol (70% v/v) until the powder id fully immersed. The flask was shaken every hour for the first 6 hours and then it was kept aside and again shaken after 24 hours. This process was repeated for 3 days and then extract was filtered.**(G. Devendran and U. Balasubramanian 2011)**

Figure number 2

Ocimum sanctum stem and leaf extract respectively.

***Ocimum gratissimum* leaf and stem extract respectively**

Figure number 3

Finally the plant extract obtained form Soxhlet was made concentrate in rotatory evaporator and kept in 15 ml tubes as shown in pic for further analysis

4.3 Spectrophotometric analysis

4.3.1 Eugenol.

Determination of λ max

The solvent system which was prepared for this determination of λ max of eugenol consists of methanol and chloroform in the ratio (of 95:5). Eugenol which was used in this used has 99 purity assay means 99 gram in 100 ml and 1000µg in 1.01 ml. Now standard eugenol 1.01 ml was taken and dissolved in 10 ml of solvent to give 100 µg in 1 ml. From these 10 ml solvent 1 ml was drawn and again dissolved in 10 ml of solvent to give 10 µg in 1 ml of eugenol. Now again form this 1 ml was taken and again dissolved in 10 ml of solvent to give 1 µg/ml eugenol. Now different aliquots were taken for determination of λ max.

4.3.2 Procedure of standard curve by using Eugenol as standard (Joshi *et al*., 2011)

1 ml of standard eugenol was dissolved in 10 ml of solvent (methanol: chloroform) to give 100µg/ml of eugenol. From this solution 1 ml was drawn and again dissolved up to 10 ml of solvent to give 10 µg/ml of eugenol. Now from 10µg/ml, 1 ml was taken and again dissolved up to 10ml of solvent to give 1 µg/ml of eugenol. Now from this final concentration different aliquots were taken and scanned. The corresponding absorbance was noted.

4.3.3 UV analysis of phenolic compound viz Eugenol in *Ocimum sanctum* and *Gratissimum*

For spectrophotometric analysis of eugenol first TLC was performed to obtain the band of eugenol and then go for UV analysis.

Ocimum sanctum L .Form thin layer chromatography the retention value of standard eugenol was compared to the band of eugenol possessed by Ocimum sanctum that was identified and scrubbed out. This scrubbed silica gel having eugenol in it was mixed with 5 ml of solvent thoroughly and filtered. Finally, this solution was taken for UV analysis.

Ocimum gratissimum L. Form thin layer chromatography the retention value of standard eugenol was compared to the band of eugenol possessed by Ocimum sanctum and was identified and scrubs out. This scrubbed silica gel having eugenol in it was mixed with 5 ml of solvent thoroughly and filtered. Finally, this solution was taken for UV analysis.

4.4 Procedure of standard curve by using chlorogenic acid as standard (Belay *et al*., 2009)

1) The reaction mixture was prepared by mixing I mg of chlorogenic acid in 1 ml of methanol.

2**)** The reaction mixture was taken and different dilution was prepared to take the readings at 324 nm.

3) The different dilutions prepared contain (5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, and 40µg/ml) respectively of chlorogenic acid

4.5 Procedure of standard curve by using gallic acid as standard (Belay *et al*., 2009)

1) The reaction mixture was prepared by mixing 1 mg of gallic acid in 1 ml of methanol.

2) The reaction mixture was taken and different dilution was prepared to take the readings at 324 nm.

3) The different dilutions prepared contain (5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, and 25µg/ml),

Respectively of gallic acid

4.6 Procedure of standard curve by using Oleanolic acid as standard (Sandra *et al*., 2009)

1) The reaction mixture was prepared by mixing I mg of oleanolic acid in 1 ml of methanol.

2) The different dilution was prepared by this reaction mixture and by subsequent addition of glacial acetic acid and 3 ml of concentrated sulphuric acid.

3) After addition of glacial acetic acid and sulphuric acid the reaction mixture was incubated at 70^0 for half an hour until the colour changes to pink shown.

4) After change in colour the reading were taken at 430 nm.

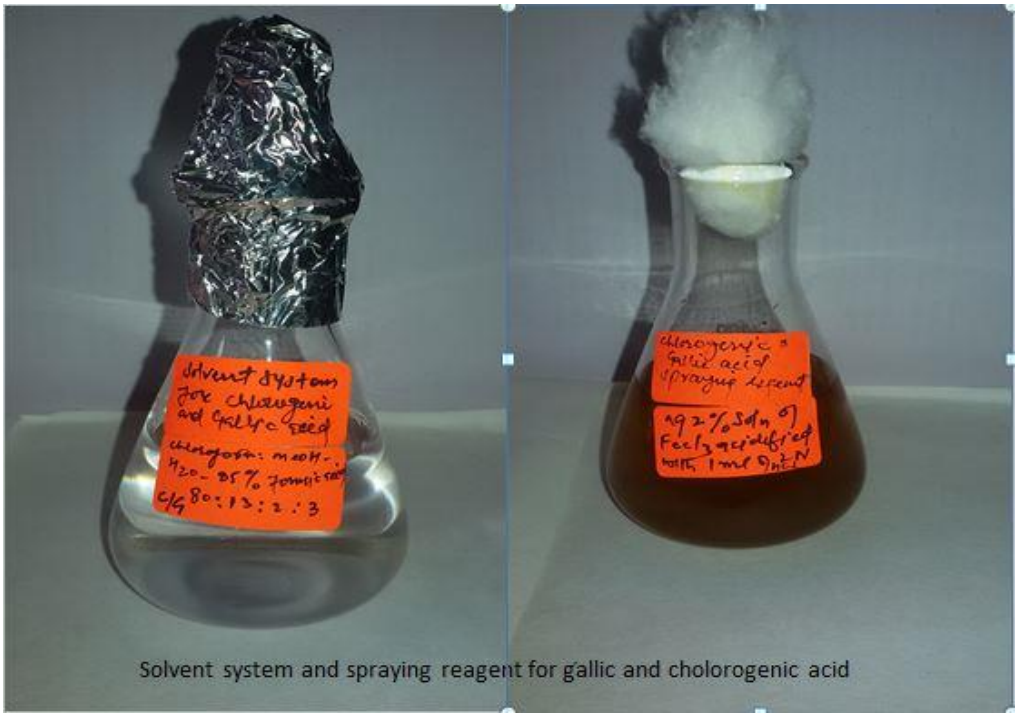
5) The different dilutions prepared were 4µg/ml, 6µg/ml, 8µg/ml, 10µg/ml, 12µg/ml and 14 µg/ ml respectively.

4.7 Thin layer chromatography

4.7.1 Qualitative analysis of Chlorogenic acid, Gallic acid, Ursolic acid, Eugenol and oleanolic acid by Thin Layer Chromatography (TLC)

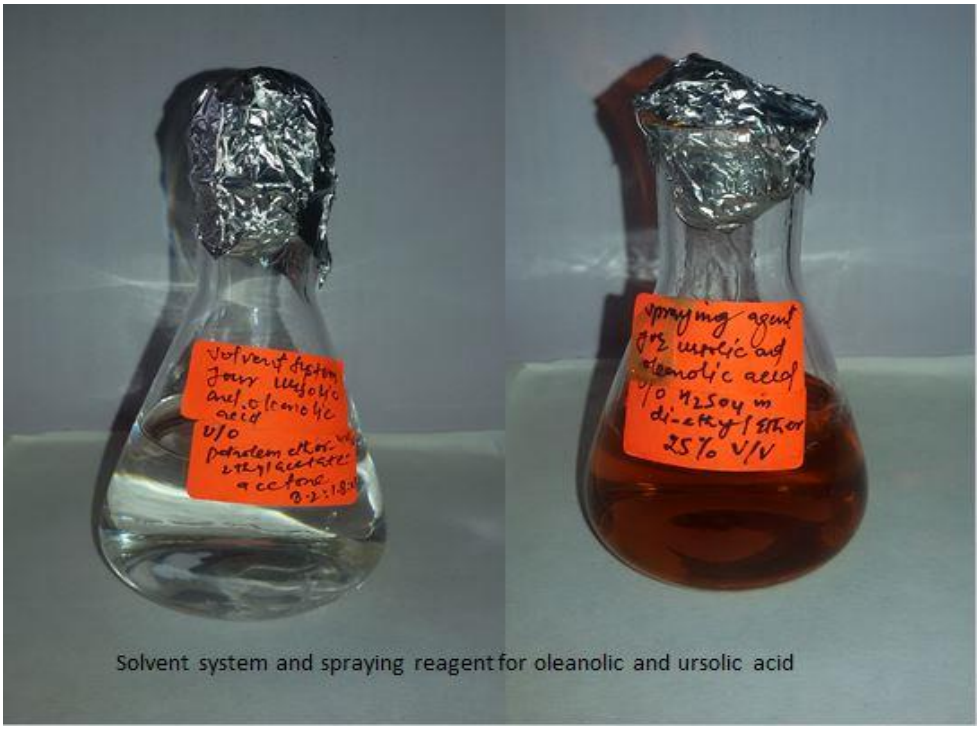
Chlorogenic and gallic acid

The detetction of chlorogenic and gallic acid was deep-rooted by TLC. Using a pencil, horizontal line was drawn about 1 cm from the bottom of the TLC plate to label the initial point for sample run.5 µl of plant extract was poured on TLC plate at the marked horizontal line and by aid of capillary tube .The plate was then kept in glass container containing solvent have different constuients viz Chloroform –Methnol-Water-85% formic acid (80:13:2:3). By capillary action, the solvent will start raising upwards the TLC plate. **(Khramov *et al*., 1998)**. As soon the solvent comes in contact with the plant sample, it will cause the less polar components of the mixture to start moving. The more polar components will also move, but at a less noticeable rate. The detection of acids occurred with an aqueous 2% solution of $Fecl_3$ acidified with 1 ml of 2N HCL .TLC plates were hand-made, the slurry was simply made and put on the plate and evenly distributed by shaking and tapering and finally the coated plates were allowed to dry about 20 minutes at room temperature and at 110^0 prior to use



4.7.2 Ursolic acid and Oleanolic acid

The presence of ursolic acid and oleanolic acid was confirmed by TLC. The detetction of chlorogenic and gallic acid was deep-rooted by TLC as shown in figure **.** Using a pencil, horizontal line was drawn about 1 cm from the bottom of the TLC plate to label the starting point for the sample run.5 µl of plant extract was poured on TLC plate at the marked horizontal line using capillary tube on this horizontal line .Then plate was then kept in glass container containing solvent have different constuients viz petroleum ether –ethyl acetate - acetone in the ratio (8.2 : 1.8 : 0.1, v/v/v).The solvent was allowed to rise on the TLC plate about 1 cm below the top of plate .The visualisation of the acid occurred by sulphuric acid in di ethyl ether (25 % v/v), by revealing pink spots on the TLC plate



Solvent system and spraying reagent for oleanolic and ursolic acid

4.7.3 Eugenol

Toluene: ethylacetate: formic acid in the ration of 3:2:0.4 (v/v/v) was prepared for eugenol separation. Using a pencil, horizontal line was drawn about 1 cm from the bottom of the TLC plate to mark the initial point of sample run. The sample spots were applied on this marked line.5 µl of plant extract was poured on TLC plate using capillary tube on this horizontal line .Then plate was then kept in glass container containing mobile phase .The solvent was allowed to rise on the TLC plate about 1 cm below the top of plate .The visualisation of the acid occurred by Phosphovanillin sulphuric acid reagent by revealing brownish spots on the TLC plate.

4.8 HPLC

Material and reagents

Quantification of eugenol, oleanolic, and ursolic acid was accomplished by means of isocratic analytical HPLC assay. It was performed on an Agilent 1260 Infinity instrument and injector with5µl loop (hypersil) and Column- DB- c18 (250×4.6 mm). Mobile phase was eluted at the flow rate of 1.5 mL/min and detected at 210 nm by photo diode array detector. Methanol, acetonitrile, Ortho-phosphoric (HPLC grade), which were utilised in this study were purchased from S.D. Fine chemicals ltd (Biosar India).Water utilised in HPLC was procured from Ranbaxy Laboratories Ltd, (Mumbai, India).Standard Ursolic acid, Oleanolic acid and Eugenol were purchased from sigma chemical company (New Delhi, India) having product code 042k1240, 093k0961 and 01050595 respectively. The peaks shown by the plant extract were identified by direct comparsion of retention time with that of standard.

4.8.1 HPLC analysis of Eugenol (vishruta *et al* ., 2013)

Preparation of Standard Solution

Eugenol Standard stock solution was prepared by dissolving 10mg in methanol and making up the volume to 10 mL with methanol. From this stock solution, standard solutions of varying concentrations were prepared by aliquots of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

Preparation of sample solution

About 250 mg of extract was taken in a 100 ml volumetric flask and boiled with sufficient amount of methanol. After this, sonication was done and the volume was made final up to 50 ml by methanol. This solution so obtained was filtered through 0.50µ membrane filter (Agilent) and finally filled in HPLC vials. The filled vials were injected in triplicate HPLC system for determination of the required phytochemicals.

Mobile phase

Methanol: Ortho-phosphoric Acid: water in the ratio of (88:0.05:11.95) was utilised for the Quantative determination of Eugenol. The chromatogram was obtained at the wavelength of 220nm and the flow rate was 1.5ml/min.

4.8.2 Ursolic acid (Gbaguidi F *et al*., 2005)

Preparation of Standard Solution

Ursolic acid standard stock solution was prepared by dissolving 10mg in methanol and making up the volume to 10 mL with methanol. From this stock solution, standard solutions of varying concentrations were prepared by aliquots of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

Preparation of sample solution

About 250 mg of extract was taken in a 100 ml volumetric flask and boiled with sufficient amount of methanol. After this, sonication was done and the volume was made final up to 50 ml by methanol. This solution so obtained was filtered through 0.50µ membrane filter (Agilent) and finally filled in HPLC vials. The filled vials were injected in triplicate HPLC system for determination of the required phytochemicals.

Mobile phase

Acetonitrile: water in the ratio of (85:15) was utilised for the Quantative determination of ursolic acid.

4.8.3 Oleanolic acid HPLC analysis (Wang H *et al*., 2008)

Preparation of Standard Solution

Oleanolic acid standard stock solution was prepared by dissolving 10mg in methanol and making up the volume to 10 mL with methanol. From this stock solution, standard solutions of varying concentrations were prepared by aliquots of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

Preparation of sample solution

About 250 mg of extract was taken in a 100 ml volumetric flask and boiled with sufficient amount of methanol. After this, sonication was done and the volume was made final up to 50 ml by methanol. This solution so obtained was filtered through 0.50µ membrane filter (Agilent) and finally filled in HPLC vials. The filled vials were injected in triplicate HPLC system for determination of the required phytochemicals.

Mobile phase

Acetonitrile: Ortho-phosphoric acid: water in the ratio of (88:0.05:11.95) was utilised for determination of Oleanolic acid in the plant samples.

4.8.4 Gallic acid HPLC analysis (Shafqatullah *et al*., 2014)

Preparation of Standard Solution

Gallic acid standard stock solution was prepared by dissolving 11mg in methanol and making up the volume to 25 mL with methanol.

Preparation of sample solution

About 500 mg of extract was taken in a 100 ml volumetric flask and boiled with sufficient amount of methanol. After this, sonication was done and the volume was made final up to 20ml by methanol. This solution so obtained was filtered through 0.50µ membrane filter

(Agilent) and finally filled in HPLC vials. The filled vials were injected in triplicate HPLC system for determination of the required phytochemicals.

Mobile phase

The optimised mobile phase was the mixture of ACN and 0.1 % aqueous acidic solution at 3.0 pH. The chromatogram was obtained at the UV wavelength of 270nm and the flow rate was set to 1.5 ml/min.

4.9 Ex-situ conservation

4.9.1 By aid of seeds

Since in the current research work we are actually confined to see the elite variety in terms of phytochemical content found in the ariel plant parts particularly leaves. So looking towards results they suggest that *Ocimum sanctum* is the elite variety. Ultimately it will be considered for ex-situ conservation. For this purpose seeds were bring from authentic source (Sheri Kashmir agricultural university). These seeds were surface sterilised with sodium hypochlorite for 5 minutes and finally inoculated in MS media. Furthermore some of the seeds were also sown in plastic trays for the purpose of ex-situ conservation.

4.9.2 Tissue culture

Leaves of *O. sanctum* have been collected and washed under running water for half an hour using tap. After washing these leaves were surface sterilised by utilizing 70% ethanol for 30 s followed by the treatment of mercuric chloride (0.1%) for 6min. After that these mercuric chloride treated leaves were washed in distilled water to remove the traces of mercuric chloride. Finally small discs were cut from these leaves and inoculated on Murashige and Skoog's medium(1962) provided with 3% sucrose, 0.9% agar and having phytohormones like 2,4-dichlorophenoxyacetic acid (2,4- D), 6-benzylaminopurine (BAP) in the concentration of 0.5mg/l and 1.0mg/l respectively for callus initiation. Prior to addition of agar pH was adjusted to 5.7 and sterilized by autoclaving for 20min having 15 psi at 121◦C. Cultures was provided 25±1◦C temperature in culture room, with light intensity of 2000–3000 lux for 16h **(C. Gopi *et al.*, 2006)**

Chapter 5

Result and discussion

5.1 Quantative estimation of Eugenol by UV

Eugenol content can be estimated and determined by UV-VIS spectrophotometer by putting the noted absorbance of eugenol from various extracts in the standard calibration curve. From the UV spectrum of standard eugenol figure number, lambda max of eugenol is 274nm

Ocimum sanctum L

Plant extract from this very plant have shown the absorbance 0.920 at near about lambda max of standard eugenol. From calibration curve it was found that 14.72 μg of eugenol present in 1 ml of solvent. Since we have 5 ml of solvent that will contain 0.0737 mg. So 1ml of solvent which contains 20 mg of extract contains 0.0737 mg of eugenol. So 5 grams of extract contains 0.0737/20×5000 mg eugenol =18.42 mg. Then the percentage of eugenol present in *Ocimum sanctum* = 18.42/5000×100=.0.36 %

Ocimum gratissimum L

Plant extract from this very plant have shown the absorbance 0.819 at near about lambda max of standard eugenol. From calibration curve it was found that 13.1 μg of eugenol present in 1 ml of solvent. Since we have 5 ml of solvent that will contain 0.0655 mg So 1ml of solvent which contains 20 mg of extract contains 0.0655 mg of eugenol. So 5 grams of extract contains 0.0655/20×5000 mg eugenol =16.37 mg. Then the percentage of eugenol present in *Ocimum gratissimum* = 16.37/5000×100= 0.32%

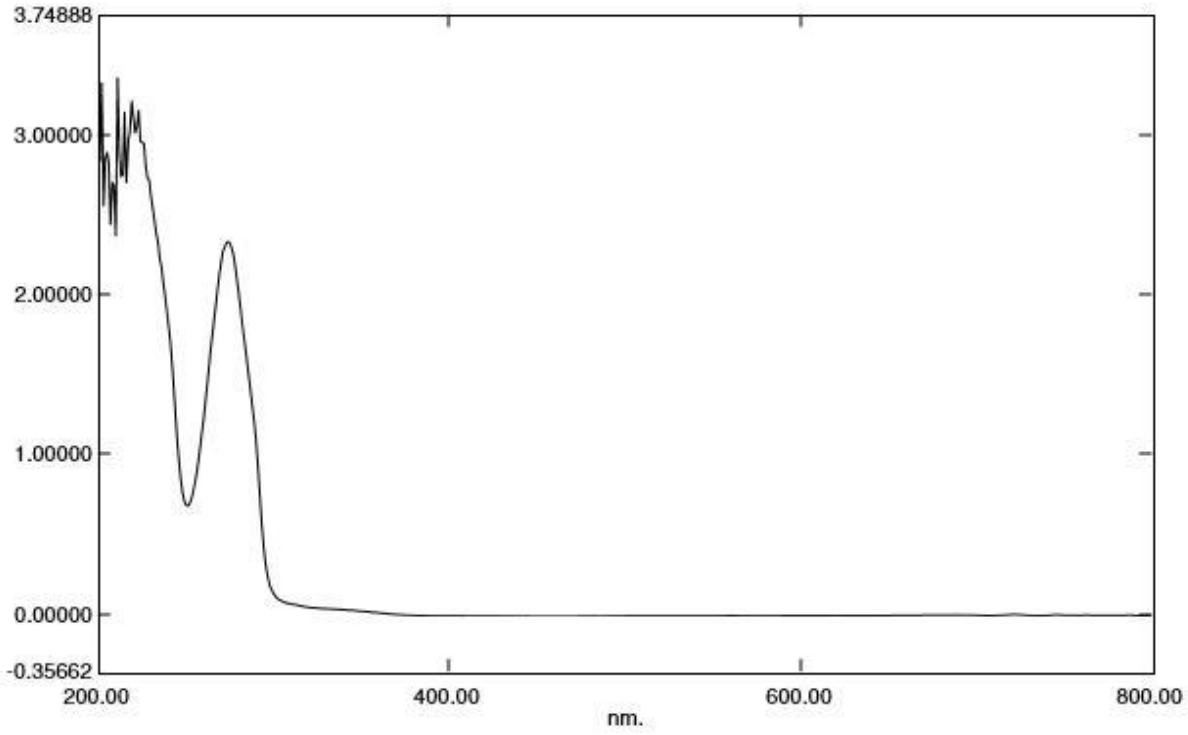


Figure 4 showing the maximum absorption of standard eugenol at 274nm

Calibration table for Eugenol (274 nm)

concentration µg/ml	Absorbance
0	0
5	0.321
10	0.620
15	0.920
20	1.271
25	1.550

Table 1 showing Calibration curve readings between concentration and absorption

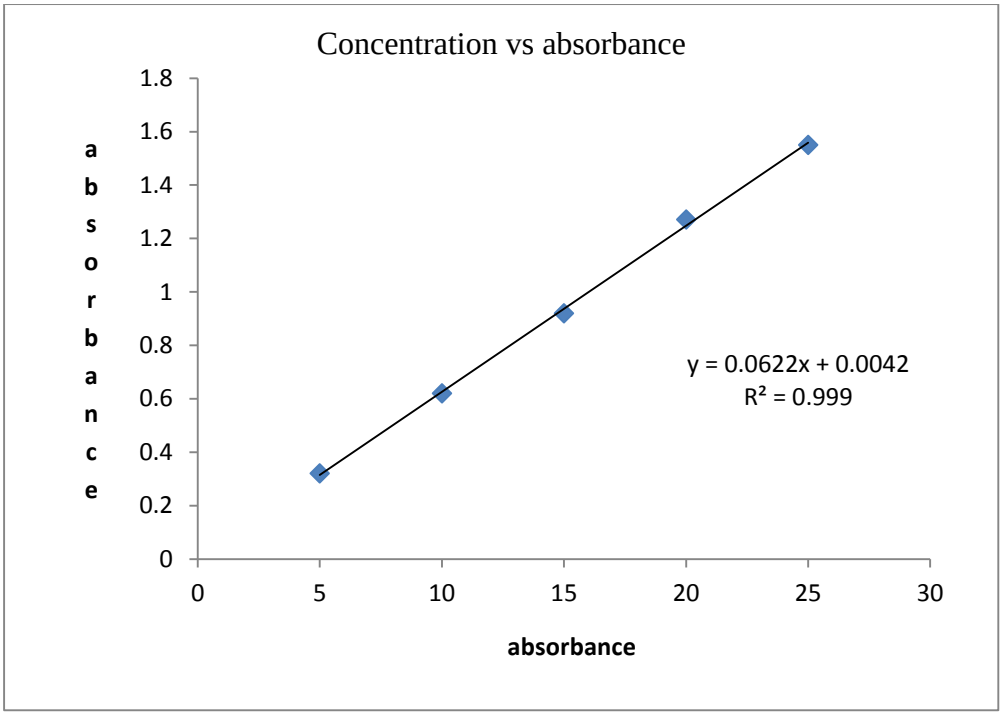


Figure 6 showing standard curve for Eugenol

5.2 Quantative estimation of Chlorogenic acid by UV

Ocimum sanctum

Plant extract from this very plant have shown the absorbance 0.290 at near about lambda max of standard chlorogenic acid. From calibration curve it was found that 7.65 µg of chlorogenic acid present in 1 ml of solvent. Since we have 5 ml of solvent that will contain 0.0361 mg. So 1ml of solvent which contains 20 mg of extract contains 0.0361 mg of chlorogenic acid. So 5 grams of extract contains 0.0361/20×5000 mg chlorogenic acid =9.025 mg. Then the percentage of chlorogenic acid present in *Ocimum sanctum* = 18.42/5000×100=.0.18 %

Ocimum gratissimum

Similarly calculating same for chlorogenic acid in *ocimum gratissimum*, It was found that plant extract have shown the absorbance of 0.420. Putting this value in regression equation we came to that it contains 0.14% of chlorogenic acid w/w

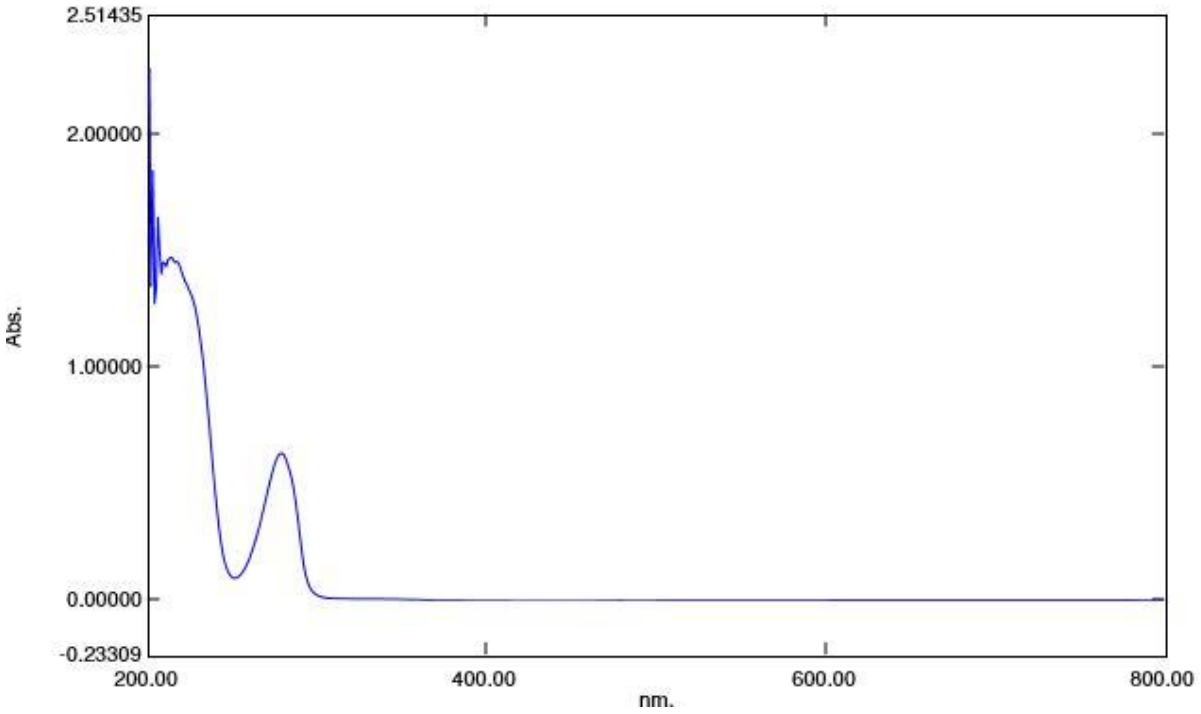


Figure 6 showing the standard chlorogenic acid maximum absorption peak at 279nm

Concentration µg/ml	Absorbance
5µg/ml	1.01
10µg/ml	1.51
15µg/ml	2.01
20µg/ml	2.30
40µg/ml	3.51

Table 2 Calibration table for chlorogenic acid (279 nm)

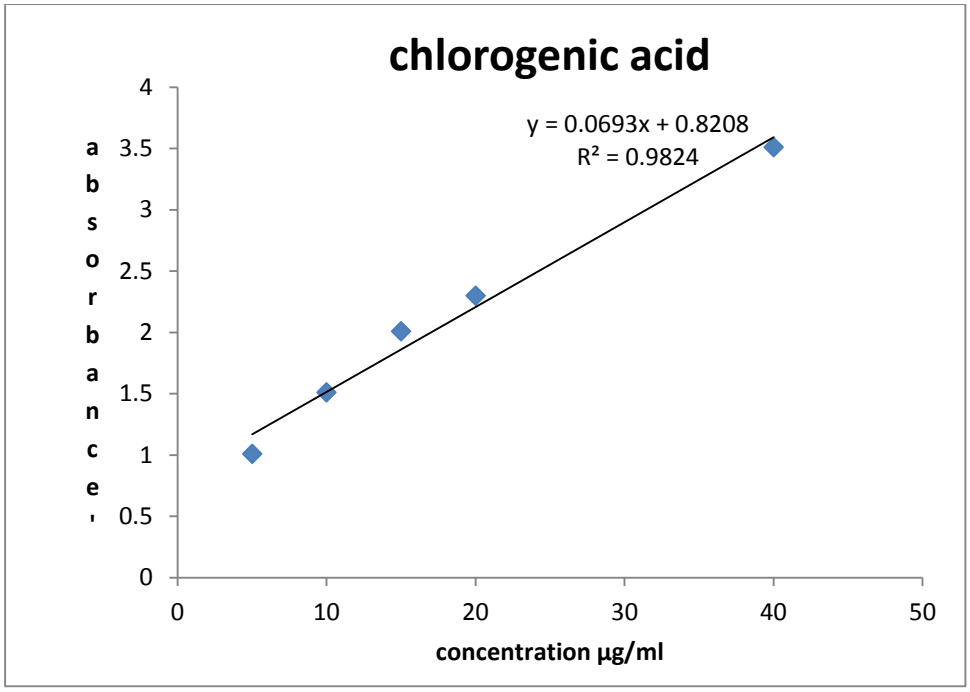


Figure 7 showing standard curve of chlorogenic acid

5.3 Quantative estimation of Gallic acid by UV

Ocimum sanctum

Plant extract from this very plant have shown the absorbance 0.620 at near about lambda max of standard gallic acid. From calibration curve it was found that 10.61 µg of Gallic acid present in 1 ml of solvent. Since we have 5 ml of solvent that will contain 0.05305 mg. So 1ml of solvent which contains 20 mg of extract contains 0.05305 mg of gallic acid. So 5 grams of extract contains 0.05305/20×5000 mg gallic acid =13.26 mg. Then the percentage of Gallic acid present in *Ocimum sanctum* = 13.26/5000×100=.0.26 %

Ocimum gratissimum

Similarly calculating same for gallic acid in *ocimum gratissimum*, It was found that plant extract have shown the absorbance of 0.430. Putting this value in regression equation we came to that it contains 0.18% of gallic acid w/w

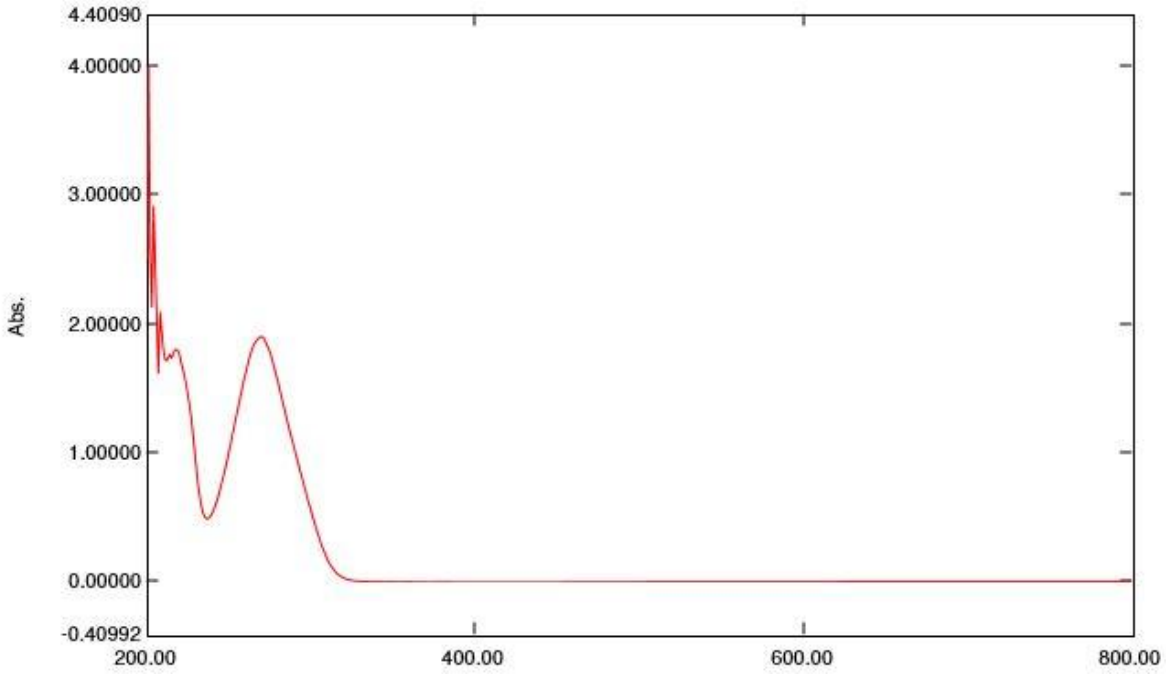


Figure 8 showing the maximum absorption shown by standard compound 269nm

Concentration µg/ml	Absorbance
5 µg/ml	0.303
10 µg/ml	0.600
15 µg/ml	0.903
20 µg/ml	1.210
25 µg/ml	1.390

Table 3 calibration for gallic acid (269nm

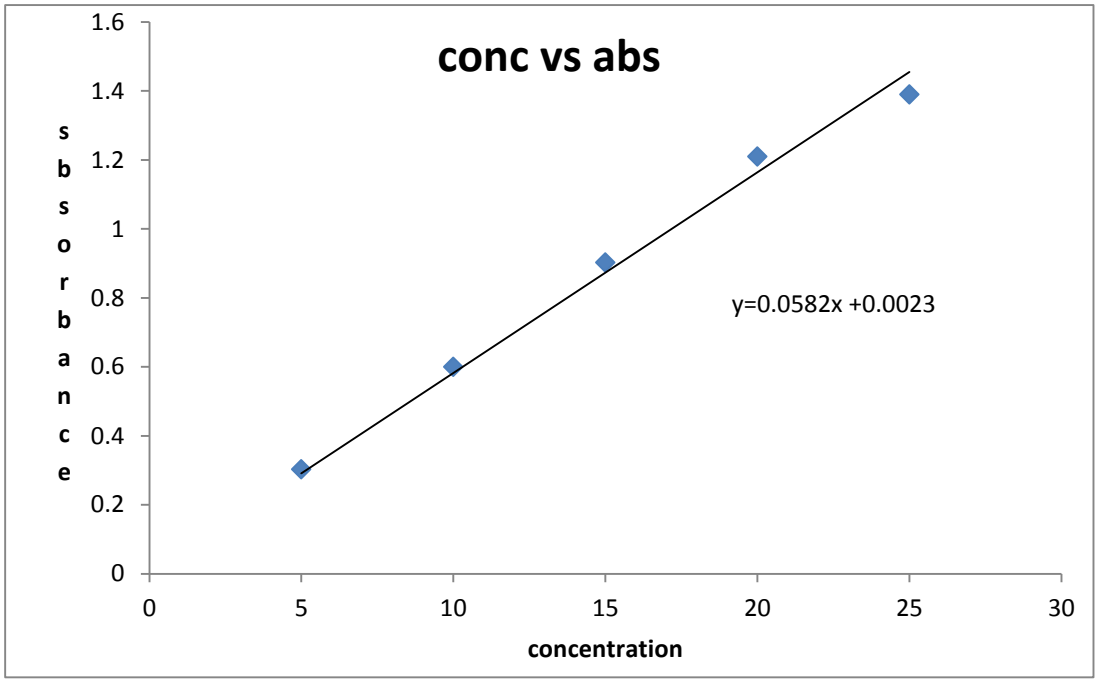


Figure 9 showing standard curve for gallic acid

5.4 Quantative estimation of Oleanolic acid by UV

Ocimum sanctum L

Plant extract from this very plant have shown the absorbance 0.220. From calibration curve it was found that 9.32 µg of oleanolic acid present in 1 ml of solvent. Since we have 5 ml of solvent that will contain 0.0466 mg. So 1ml of solvent which contains 20 mg of extract contains 0.0466 mg of OA. So 2 grams of extract contains 0.0466/20×2000 mg oleanolic acid =4.66 mg. Then the percentage of oleanolic acid present in *Ocimum sanctum* = 4.66/2000×100= 0.23 %

Ocimum gratissimum L

Plant extract from this very plant have shown the absorbance 0.194. From calibration curve it was found that 8.04 µg of Oleanolic acid present in 1 ml of solvent. Since we have 5 ml of solvent that will contain 0.040 mg. So 1ml of solvent which contains 20 mg of extract contains 0.040 mg of OA. So 2 grams of extract contains 0.040/20×2000 mg OA=4 mg. Then the percentage of oleanolic acid present in *Ocimum gratissimum*= 4/2000×100= 0.20 %

4 µg/ml	0.11
6 µg/ml	0.15
8 µg/ml	0.20
10 µg/ml	0.23
12 µg/ml	0.27
14 µg/ml	0.32

Table 4 Calibration readings for Oleanolic acid (430 nm)

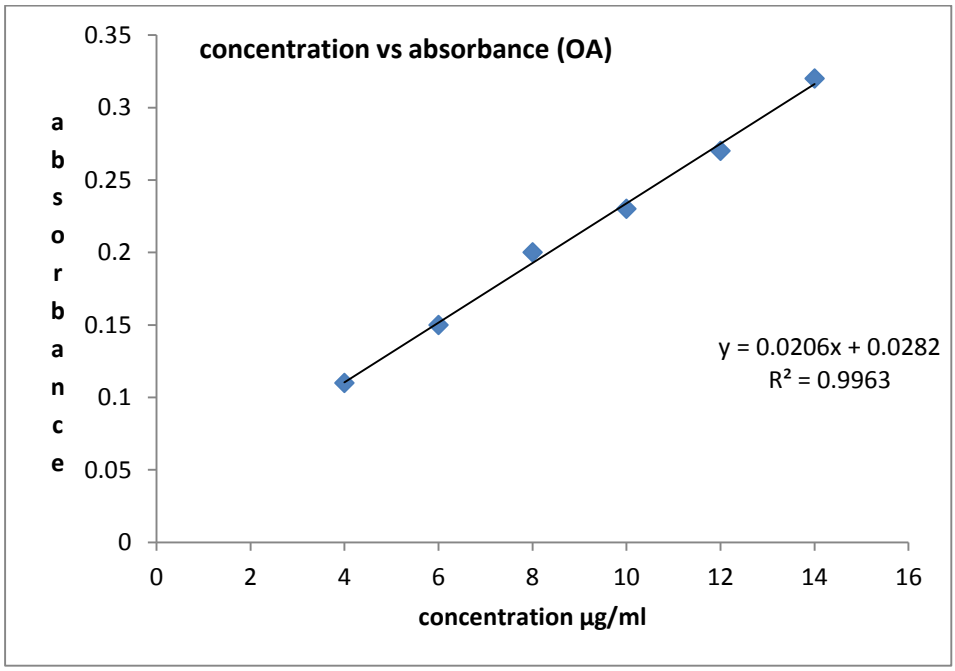


Figure 10 showing standard curve of oleanolic acid

Table 5 Spectrophotometric analysis in *Ocimum gratissimum*

Compound	Sample O .D	Equation	R^2	percentage (w/w)
Eugenol	**0.920**	**y=0.0622x+0.0042**	**0.999**	**0.32%**
Gallic acid	**0.430**	**y=0.0582x+0.0023**	**0.982**	**0.18%**
Chlorogenic acid	**0.420**	**y=0.0693x+0.8208**	**0.999**	**0.14%**
oleanolic acid	**0.194**	**y=0.0206x+0.0282**	**0.996**	**0.20%**

Table 6 Spectrophotometric analysis in *Ocimum sanctum*

Compound	Sample O.D	Equation	R^2	Percentage (w/w)
Eugenol	**0.819**	**y=0.0622x+0.0042**	**0.982**	**0.36%**
Gallic acid	**0.620**	**y=0.0582x+0.0023**	**0.998**	**0.26%**
Chlorogenic acid	**0.290**	**y=0.0693x+0.8208**	**0.999**	**0.18%**
oleanolic acid	**0.220**	**y=0.0206x+0.0282**	**0.996**	**0.23%**

From the spectrophotometric analysis of the two undertaken *Ocimum* species, it is clear from the results that *Ocimum sanctum* is elite variety in terms of pharmacologically active constuients. From table number 6 and 7 it can be visualised that which phytochemical possess what amount of in two said plants. This difference is mainly attributed to the fact that in plants different geographical variations do affect the phytochemical constuients. Besides, this time of collection, growth stage of plants also affect the phytochemical constuients.

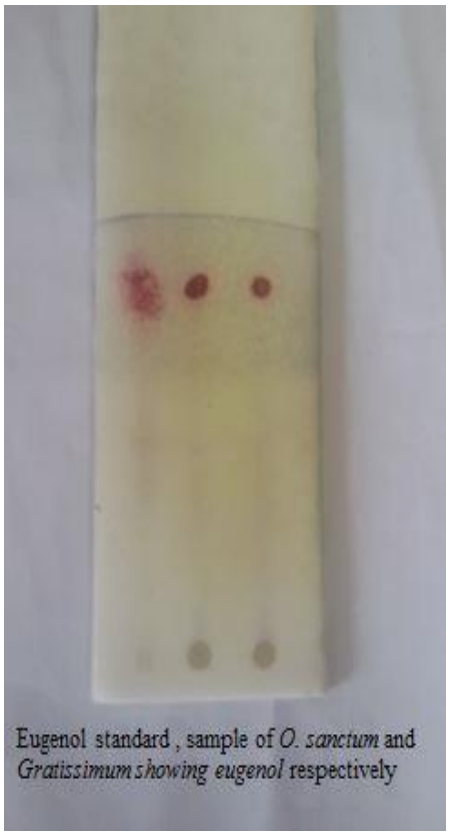
5.5 Thin layer chromatography

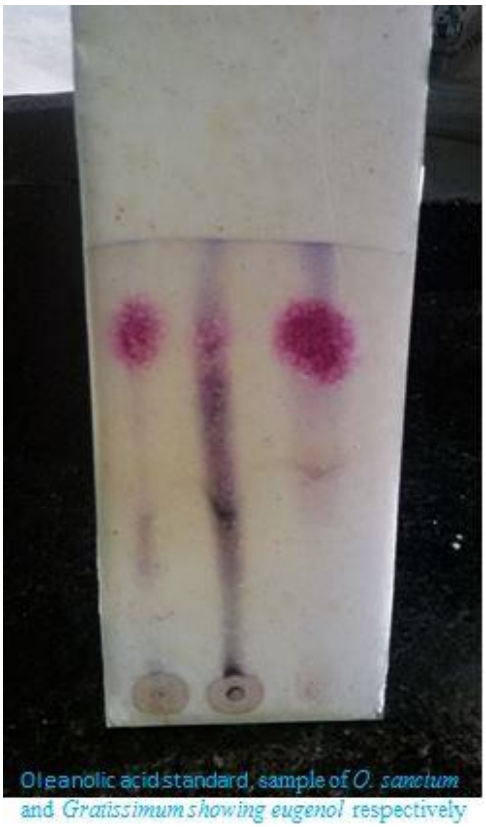
R_f value of chlorogenic acid and Gallic acid on TLC plates			
S .no.	**Acid**	**R_f**	**Colour reaction with $Fecl_3$**
1	Chlorogenic acid	0.6	Olive green
2	Gallic acid	0.11	Violet

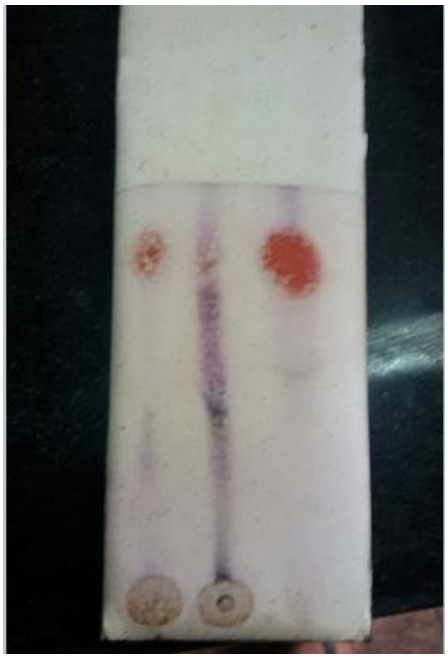
Table 7 showing the Rf values of chlorogenic and gallic acid respectively

R_f value of Ursolic acid and oleanolic acid on TLC plates			
S .no	**Acid**	**R_f**	**Colour**
1	Ursolic acid	0.14	Pink
2	Oleanolic acid	0.29	Pink
3	Eugenol		

Table 8 showing the Rf values of ursolic and oleanolic acid obtained with TLC







Ursolic acid **Chlorogenic acid**

Figures 11 showing the TLC plates of different compounds

5.6 HPLC analysis of eugenol

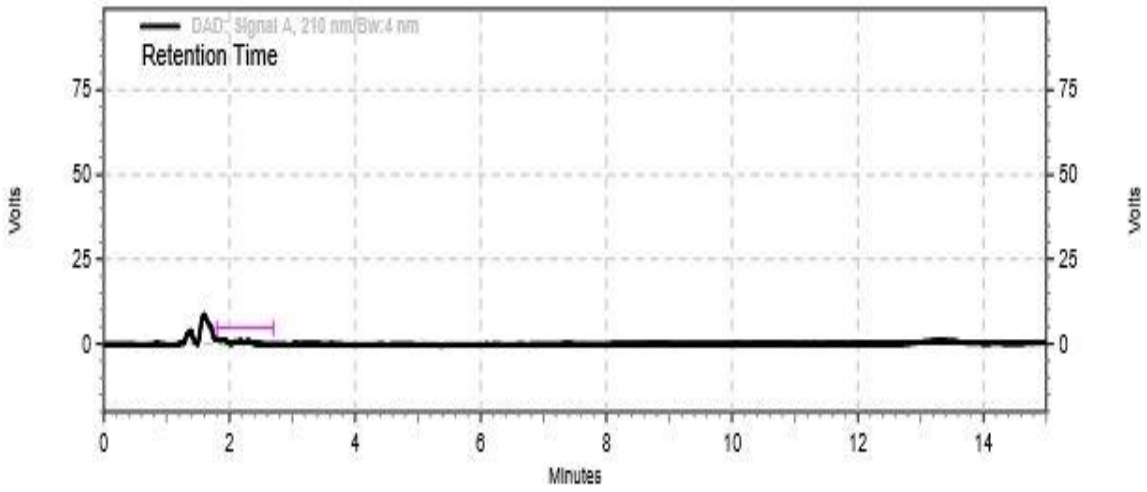


Figure 12 showing blank run on HPLC instrument ist to calibrate

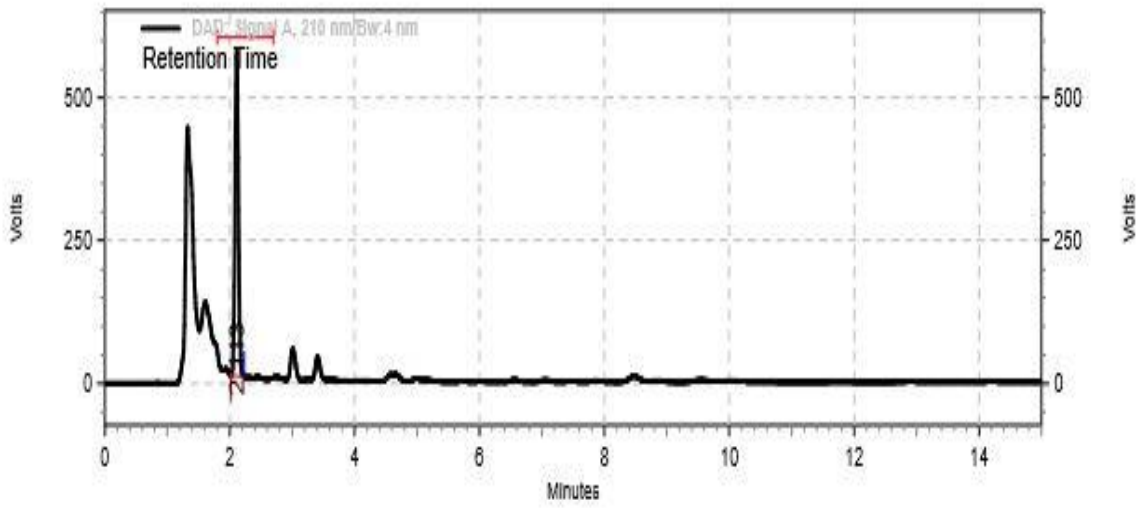


Figure 13 showing the standard eugenol run in HPLC with retention time of 2.113

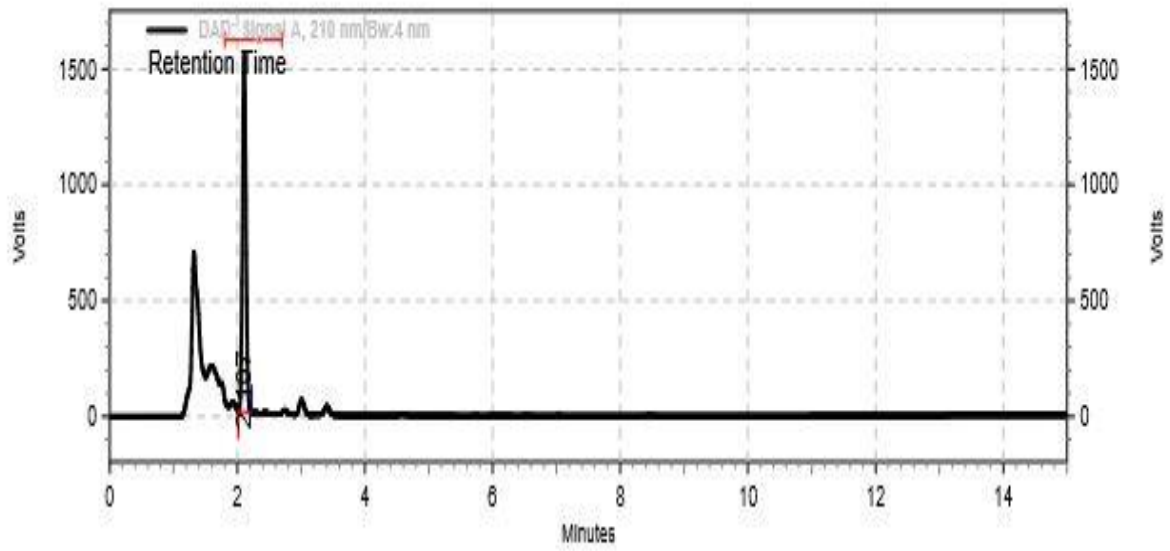


Figure 14 showing eugenol in the methanolic extract of *Ocimum sanctum* with same retention time

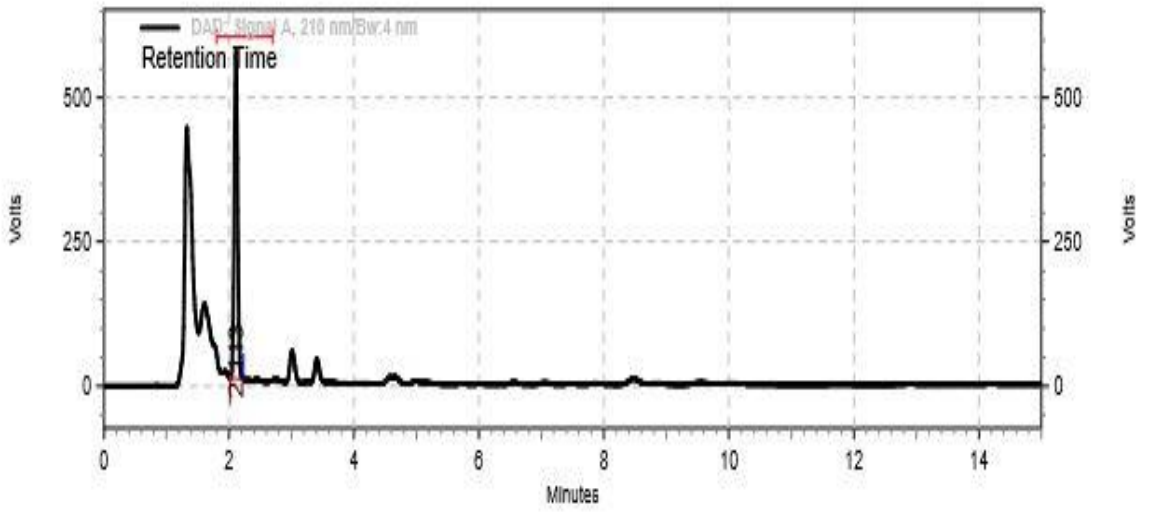


Figure 15 showing Eugenol in the methanolic extract of *Ocimum gratissimum*

The already developed method facilitates the determination of Eugenol in *Ocimum sanctum* and *Ocimum gratissimum* respectively. The wave length of detection utilised for eugenol was 210 nm. Quantification of this very analyte in the leaf extract of these two species are summarised in the table number 11.

5.7 HPLC analysis of Gallic acid

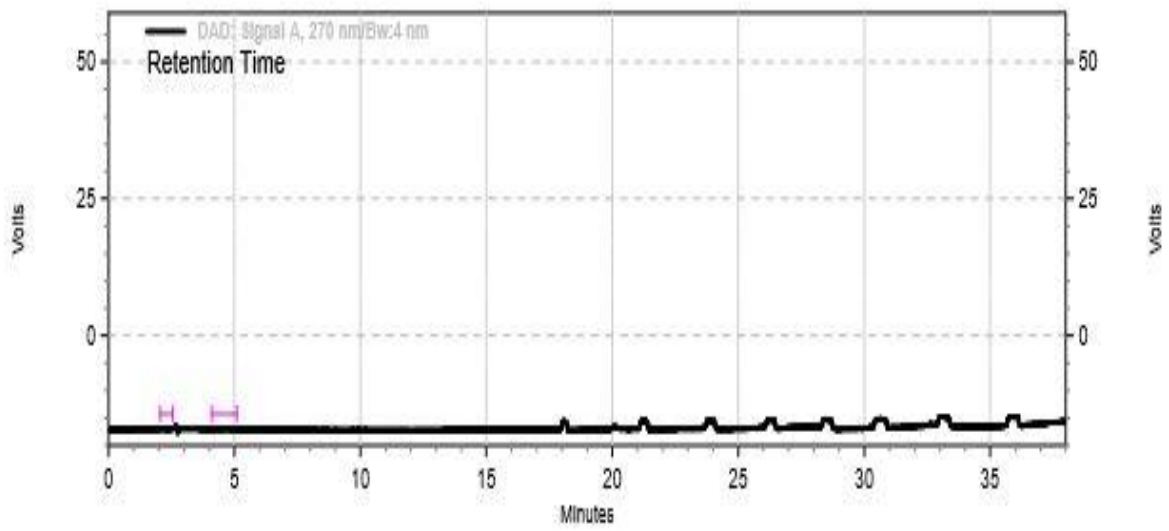


Figure 16 showing blank run on HPLC instrument ist to calibrate

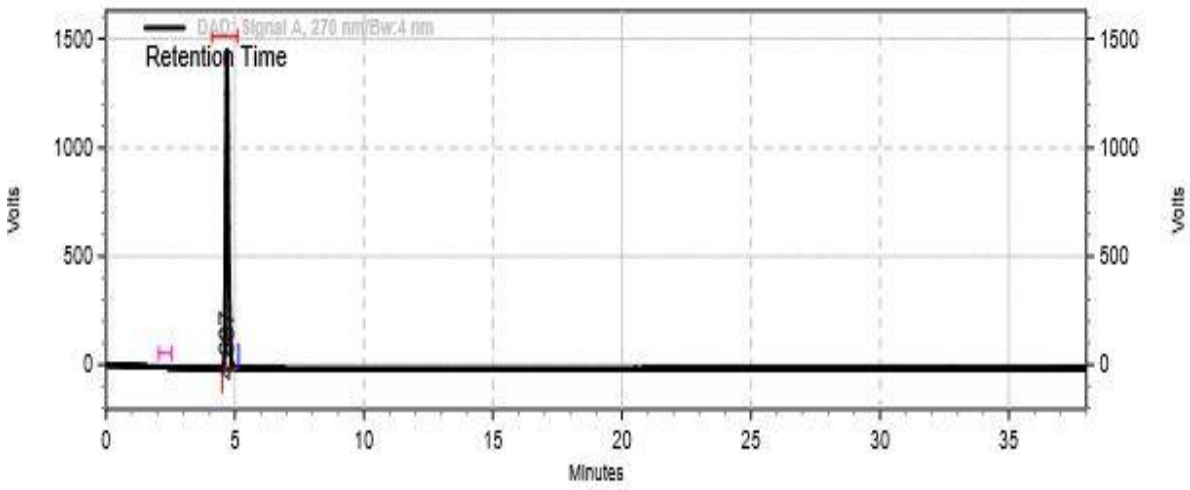


Figure 17 showing the standard gallic acid run in HPLC with retention time of 4.687

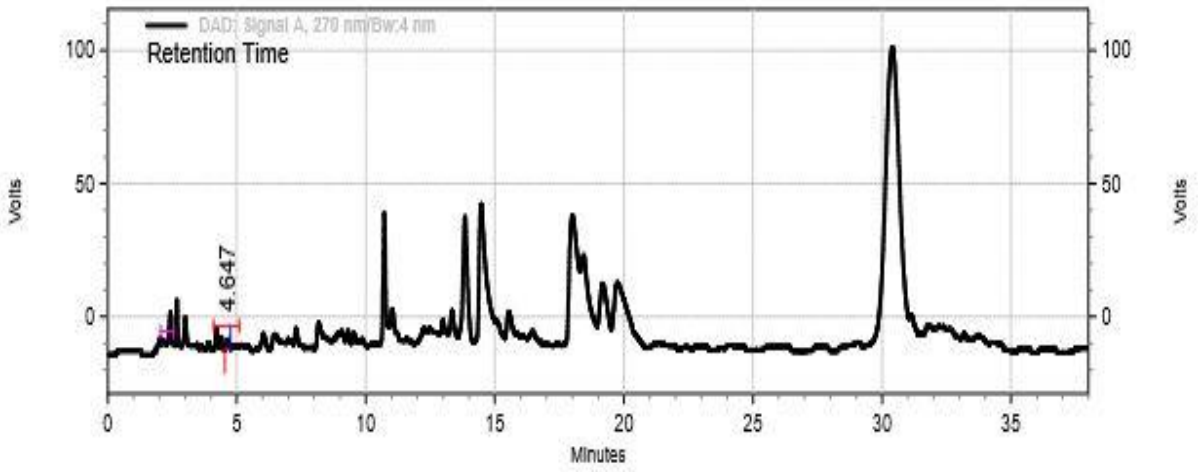


Figure 18 showing Gallic acid present in extract of *Ocimum sanctum*

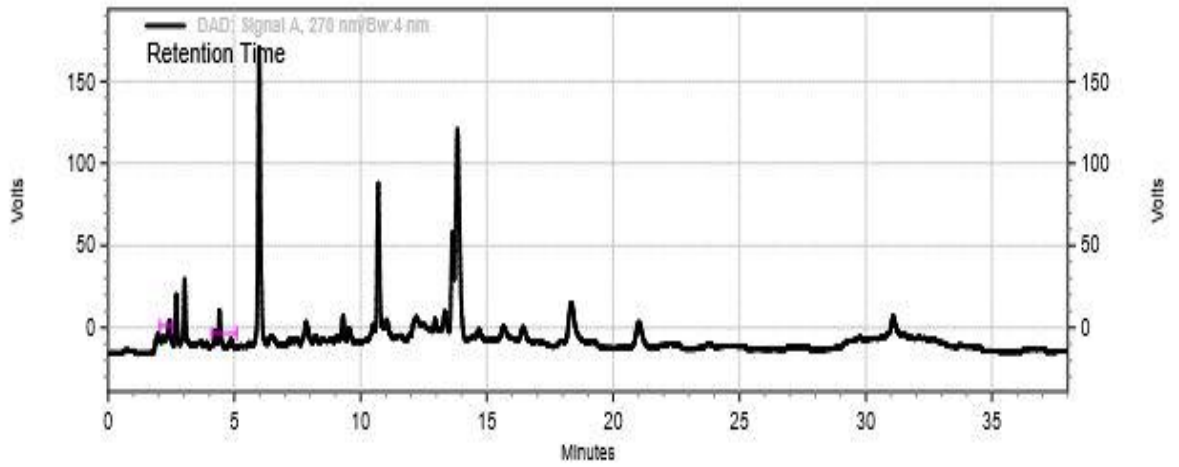


Figure 19 showing Gallic acid present in extract of *Ocimum gratissimum*

5.8 HPLC analysis of Oleanolic acid

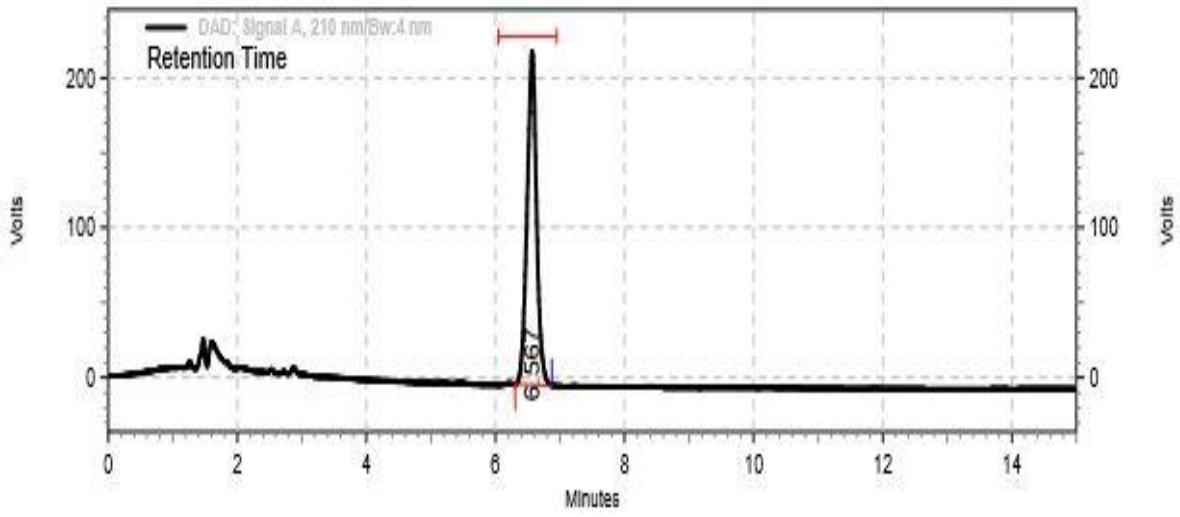


Figure 20 showing the standard oleanolic acid run in HPLC with retention time of 6.576

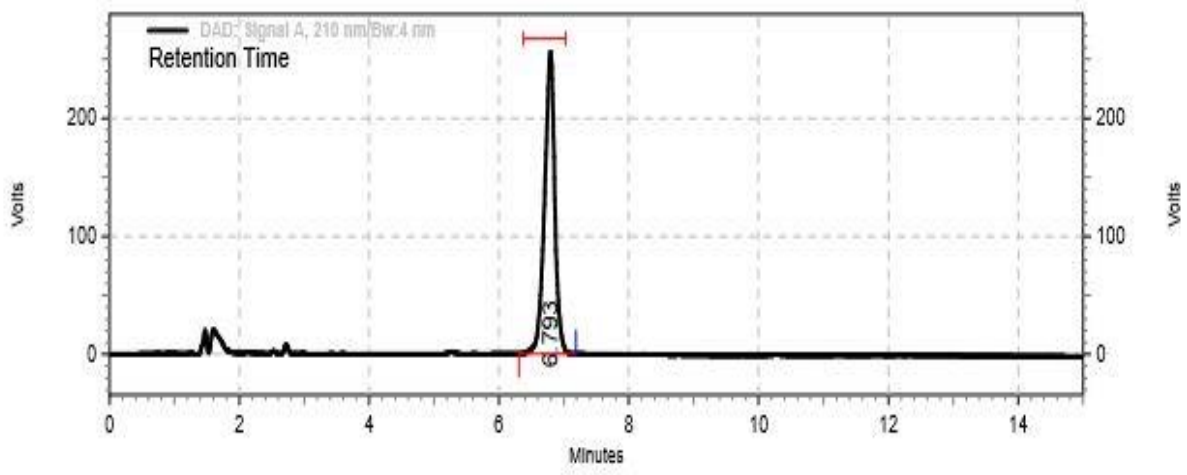


Figure 21 showing the standard ursolic acid run in HPLC with retention time of 6.793

Analyte (standard)	Retention time	Peak area	Purity of Compound
Eugenol	2.113	36373614	99 %
Gallic acid	4.687	22277759	99.5 %
Oleanolic acid	6.567	4759955	98.5 %
Ursolic acid	6.793	5871332	99%

Table 9 showing the retention time and peak area of above mentioned compounds

S.no.	Analyte	*Ocimum sanctum*		*Ocimum gratissimum*	
		RT	Peak area	RT	Peak area
1	Eugenol	2.107	12131175	2.113	4416854
2	Gallic acid	4.647	48495	4.645	46540

Table 10 showing the retention time and peak area of two different plant extracts

S. no.	Analyte	Concentration of terpenoids and phenolics in plant extracts(leaves) w/w	
		Ocimum sanctum	*Ocimum gratissimum*
1	Eugenol	5.985%	2.179%
2	Gallic acid	0.09%	0.0037%

Table 11 showing quantification results by HPL

5.9 Ex-situ conservation

For ex-situ conservation of *Ocimum sanctum* two things were taken in consideration viz to grow plants by utilising seeds and to go for callus initiation form leaf explants. Furthermore seeds were sown in plastic trays as well inoculated directly on MS media by giving them first sterilisation treatment. According to previous research, they have shown that seeds germinate after one week but unfortunately seeds inoculated on MS media didn't show any response of germination. However, seeds sown in plastic trays show germination after 11 days. Picture number 22 shows the seedlings growing in plastic trays. These seedlings are one month old.

Figure 22 shows the seedlings growing in plastic trays

5.9.1 Tissue culture

From Micropropagation point of view explants taken from leaf have shown good response and have taken short duration of time for callus initiation followed by stem which has taken more time for callus initiation. Different concentration of hormones was utilised for both these parts for callus initiation and subsequent sub culturing. Duration of time for different explant and their response in shown in the table number 12 for *Ocimum sanctum.*

Tissue culture analysis table

Explant	Ms media	Growth hormones	Result	Callus initiation		Time duration
1) Leaf	Full strength	2,4D,BAP, IAA	Callus formation	+		3 weeks
2) Stem	Full strength	2,4D,BAP, IAA	Callus formation	+		1 month
3) Callus (sub culturing)	Full strength	BAP, IAA	Enlargement of callus	+		9 days
4) Sub parts of callus	Full strength	BAP	Enlargement of callus	+		3 weeks

Table 12 showing the result of in vitro culture performed on *Ocimum sanctum*

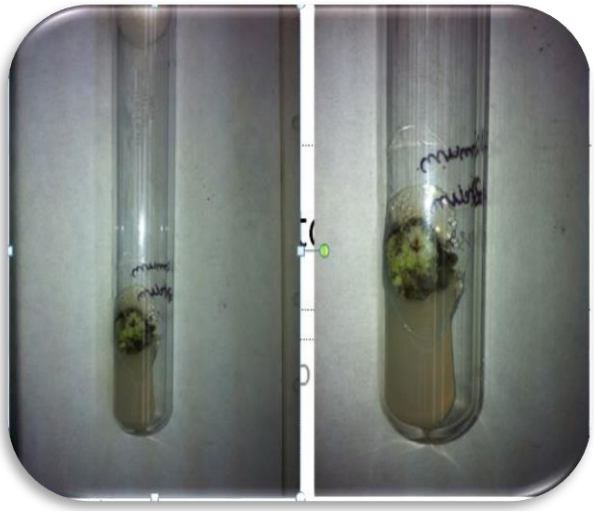


Figure 23 showing callus initiation from leaf explant

Figure 24 showing further callus growth

Chapter 6

Conclusion and future scope

The principle aim of the present work is to study Quantitative and Quantative analysis of bioactive compounds in *Ocimum sanctum* and *Ocimum gratissimum* samples collected from different geographical locations in India was carried out to evaluate variability in bioactive

compound content and to select elite variety sample for ex- situ conservations. The protocol established in this study will enable future works on developing *Ocimum sanctum* propagating systems. Theses would enable the development of genetic transformation protocols to address concerns about diseases and pest resistance, and also to start a biochemical selection, focussing secondary metabolites production. Secondary metabolites are specific to an individual species or genus during specific environmental condition making their extraction and purification difficult. As a result, commercially available secondary metabolites (pharmaceuticals, flavours, fragrances and food additives) are generally considered high value products as compared to primary metabolites. *Ocimum sanctum* has lot of the medicinal values and has got religious importance as well besides this the plant secondary metabolites for anti-infective agents are important because of increase in the rate of resistance of pathogenic microorganisms to existing antibiotics. So, the need to develop efficient, safe and inexpensive drugs from plant resources is of great importance.

Furthermore to screen elite variety in terms of phytochemicals has lot of benefits like it will generate good economy for farmers who are actually actively involved in its farming as they will be able to grow elite variety. Beside this pharmaceutical companies would easily identify elite and inferior variety among different medicinal plants.

Chapter 7

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