"COMPARATIVE PHARMACOGNOSTICAL AND PHYTOCHEMICAL EVALUATION OF VARIOUS SPECIES OF GENUS OCIMUM"

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BY

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CERTIFICATE

This is to certify that **Mr. Sushil Kumar Aggarwal** has prepared his thesis entitled "**Comparative pharmacognostical and phytochemical evaluation of various species of genus ocimum**", in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under our guidance. He has carried out the work at the Department of Phytopharmaceuticals and Natural products, Institute of Pharmacy, Nirma University.

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I declare that the thesis "***Comparative pharmacognostical and phytochemical evaluation of various species of genus ocimum***" has been prepared by me under the guidance of **Dr. Sanjeev R. Acharya,** Associate Professor and **Dr. Niyati S. Acharya,** Assistant Professor, Department of Phytopharmaceuticals & Natural Products, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones are not a house and a collection of facts is not necessarily science.

*There is a famous saying "**Work always Works**". There is always the danger that we may just do the work for the sake of the work. This is where the respect, love and the devotion come in - that we do it to God, to Christ and that's why we try to do it as beautifully as possible.*

Education is not the amount of information that is put into your brain and runs riots there, undigested, all your life. Therefore, we must have life building, man making, character building, and assimilation of ideas.

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"If I can see farther it is only because I stand on the shoulders of giants"

Date:

Place: Ahmedabad ***Sushil Kumar Aggarwal***

CONTENTS

LIST OF ABBREVIATIONS

WHO	World Health Organization
TLC	Thin Layer Chromatography
HPTLC	High Performance Thin Layer Chromatography
UV	Ultra Violet
MIC	Minimum Inhibitory Concentration
MTCC	Microbial Type Culture Collection
DMSO	Dimethyl Sulfoxide
BOD	Biological Oxygen Demand
DPPH	α, α Diphenyl–β Picryl Hydrazyl
µg	Micro Gram
gm	Gram
µl	Micro Liter
mg.	Mili gram
mm	Mili Meter
ppm	Part Per Million
hr.	Hour
temp.	Temperature
T.S.	Transverse Section
N	Normality
nm	Nanometer
v/v	Volume/Volume
R_f	Retention factor
WFI	Water for injection
DPPH	1,1-diphenyl-2-picrylhydrazyl
p	Para
lit.	Liter
std.	Standard
conc.	Concentration

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3. AIM OF WORK

The genus Ocimum is a well known genus comprised of strongly scented aromatic herbs, under shrubs and shrubs. A lot of work had been done on various species belonging to this genus. However, the best of our knowledge, comparative studies on various species have not been reported so far. Hence the aim of current work is to establish some comparative pharmacognostical and phytochemical data amongst various species of genus Ocimum like *O.sanctum* (Black variety), *O.sanctum* (Green variety), *O.basilicum* and *O.citriodorum* so that they can be distinguished from each other easily.

However, our earlier aim was to isolate oleanolic acid from black variety of *Ocimum sanctum*. According to one report it was claimed to contain 0.17% of oleanolic acid. (Anandjiwala et al 2006). But in our experimental work it was not been isolated from this plant inspite of much efforts done. In literature *O.sanctum* is reported to have two varieties black and green but in all the reports for *O.sanctum* there are no sufficient published information about the variety used in the reported studies. In the light of above scenario, our interest was develope to conduct comparative pharmacognostical and phytochemical study between these two varieties of *O.sanctum*. We also included two more species *O.basilicum* and *O.citriodorum* in our present work.

Morever there is scanty literature records on pharmacognostical work on the leaves of *O.citriodorum*, hence the present work is undertaken to produce some pharmacognostical standards for this species. Extensive pharmacognostical work on *O.citriodorum* would be carried out and various parameters would be established for this species including HPTLC finger printing of volatile oil for these four species of Ocimum. In current study, we also aimed to carry out some comparative evaluation on certain pharmacological activities like antibacterial, antifungal and antioxidant activity.

Scientific parameters are yet not available to identify the true plant material from its allied species of genus Ocimum and to ensure its quality. Therefore the present work

has been also undertaken to establish the necessary pharmacognostical standards for evaluating the plant material from its allied species. Various parameters like morphology, microscopy, physio-chemical constants and phytochemical tests, fluorescence analysis etc. would be studied and the salient diagnostic features would be documented. Further with the use of HPTLC method finger printing for volatile oil obtained from all four species would be established.

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Nature provided man with all the basic requirements for his existence. The early civilizations valued nature and nature worship was common in those times. After coming in contact with plants, people began to realize their significance. Nature always stands as golden marks to exemplify the outstanding phenomena of symbiosis. In the western world, people are becoming aware of the potency and side effect of synthetic drugs. So there is an increasing interest in the natural remedies with a basic approach towards the nature. In India great deals of in depth knowledge exist among general public about the traditional use of herbal medicine. This is in addition to organized Indian system of medicine which has already gained worldwide attention.[1]

Plants are an integral part of human life from time immemorial. Most of the present day drug molecules were derived from the active molecules found in the plants of traditional systems of medicine. Plants are a tremendous source for the discovery of new products of medicinal values for drug development. Several distinct chemicals derived from plants are important drugs currently used in different parts of the world. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances.[2]

Different parts of these medicinal plants have been reported to be effective in various diseases. In several traditional medicinal system, including Ayurveda, Roman, Greek, Sidha and Unani medicinal system, various therapeutic properties of these plants have been mentioned. They are reported to posses antibacterial, antifungal, analgesic, anthelmintic, antidiabetic, antipyretic, larvicidal, antioxidant, cardio tonic, hepatoprotective, antiulcer activity etc.[3]

Medicinal plants have played an important role throughout the world in treating and preventing human diseases. Plants are the primary sources of medicines since ancient times and continue to provide mankind with new remedies. The belief that plant based natural medicines are much safer than synthetic drugs has gained popularity in recent years and led to tremendous growth of phytopharmaceutical usage.[4]

Plants are a tremendous source for the discovery of new products of medicinal values for drug development. Several distinct chemicals derived from plants are important drugs currently used in different parts of the world. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances.

Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Research in bioactive substances from plants might lead to the discovery of new compounds that could be used to formulate new and potent drugs.

Medicinal values of plants and herbs are immense and they are recommended for various ailments. These days a lot of work is going on various medicinal plants. Comparative pharmacognostical and phytochemical study is important work for these medicinal plants so that they can be easily distinguished from each other.

1.2 INTRODUCTION TO FAMILY LABIATAE

The Labiatae family (Lamiaceae) is one of the largest and most distinctive families of flowering plants. This family has an almost cosmopolitan distribution.The family Labiatae or Lamiaceae, also known as the mint family. It has been considered closely related to family verbenaceae. Lamiaceae contains 263 genera and 7200 species.

The original family name is Labiatae, so given because the flowers typically have petals fused into an upper lip and a lower lip. Many members of this family are used in traditional and folk medicine. Also they are used as culinary and ornamental plants. Labiatae are best known for the essential oils common to many members of the family. Many biologically active essential oils have been isolated from various members of this family.

Labiatae are best known for the essential oils common to many members of the family. Many biologically active essential oils have been isolated from various members of this family. The family is also famous for the presence of diterpenoids in its members. These plants have been used by humans since prehistoric times. Evidence from

archeological excavations shows that some species of this family, which are now known only as wild plants, had been cultivated at local scales in the past. This family is one of the major sources of culinary, vegetable and medicinal plants all over the world. Species of Mentha, Thymus, Salvia, Origanum, Coleus and Ocimum are used as food flavorings, vegetables and in industry. [1,2,5,6]

Following are the characteristics of this family:

- The leaves emerge oppositely, each pair at right angles to the previous one called decussate or whorled.
- The stems are frequently square in cross section.
- The flowers are bilaterally symmetrical with 5 united petals and 5 united sepals. They are usually bisexual and verticillastrate. Verticillastrate is a flower cluster that looks like a whorl of flowers but actually consists of two crowded clusters.

The following are the some genera in this enlarged family:

- Vitex
- Ocimum
- Salvia
- Thymus
- Tinnea
- Mentha
- Lycopus
- Bovonia
- Coleus

1.3 INTRODUCTION TO GENUS OCIMUM

The genus Ocimum, collectively called as basil. It contains at least 60 species and numerous varieties. The name basil is derived from the Greek word *basileus,* meaning "king" because of its wonderful "royal" fragrance. The French call it "herb royale." In ancient times, basil was considered to have magical properties. The Greeks gave basil the name *basilisk* because it was reputed to provide protection from a half-lizard, half-dragon monster of the same name.[7]

It is a genus of aromatic herbs, under shrubs and shrubs. It is distributed in the tropical and warm temperate regions of Asia, Africa, and Central and South America. Basil originated from Iran, India and other tropical regions of Asia, having been cultivated there for more than 5,000 years. In India, all of the basils are honoured as Tulsi and belonging to the family *Lamiaceae /Labiatae* (mint family).

It offers a wide diversity among its more than 50 species, particularly regarding plant growth, morphology, physical appearance and essential oil content and composition.

The nomenclature of Ocimum species and varieties is complicated and confused and it is difficult to classify the oils according to the botanical nomenclature of plants from which they are derived. Nine species of Ocimum are found in india of which three are exotic. Several Ocimum species yield essential oils which are valued in medicines and perfumery, a few are rich source of camphor. Some Ocimum species are widely used in traditional medicine for different applications such as analgesic, antitussive medicine and corpse preservative especially in many Asian and African countries.[8, 9]

Following are few of the species in genus Ocimum.

- *Ocimum sanctum* (Syn. *O. tenuiflorum*), (Sacred/holy basil)
- *O. americanum* (Syn. *O.canum*) (Hoary Basil)
- *O. basilicum* (Sweet Basil),
- *O. gratissimum* (Shrubby Basil/ Vana Tulsi)
- *O. citriodorum* (Lemon basil)

- *O. campechiamum* (Peruvian basil),
- *O. minimum* (Greek basil),
- *O. kilimandscharicum* (Camphor basil),
- *O. viride*

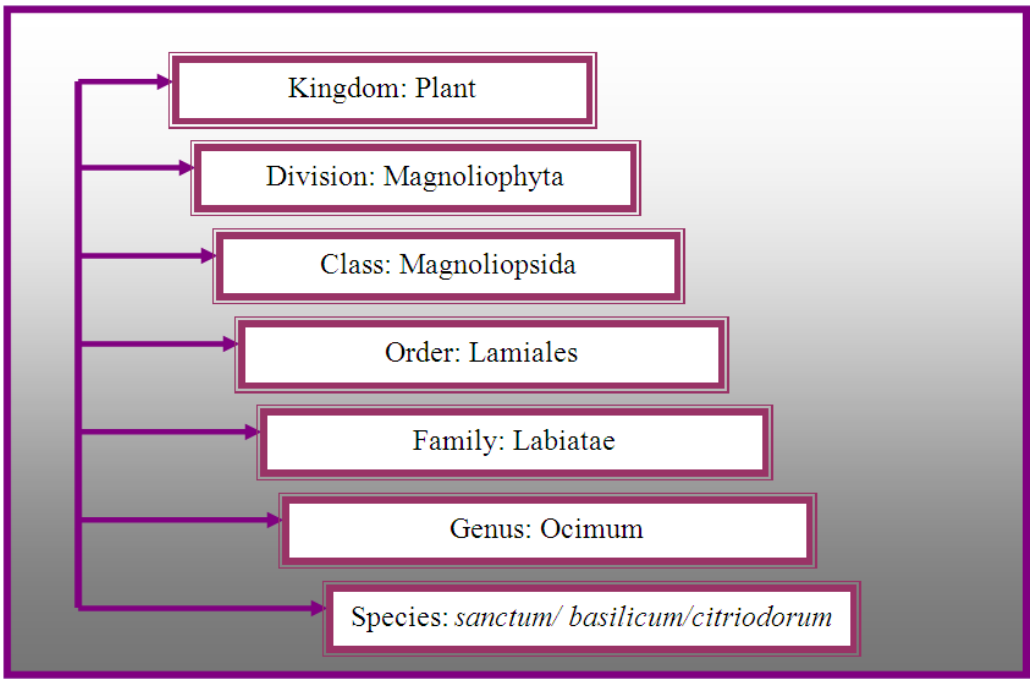
Basil is one of the species used for the commercial seasoning. It is commonly known that the presence of essential oils and their composition determine the specific aroma of plants and the flavour of the condiments. Basil folklore is as complex as its flavour and aroma. They are heat and drought tolerant i.e. they do not require high humidity. Basil is a source of essential oils and aromatic compounds, culinary herb, and an attractive fragrant ornamental. The seeds also contain edible oils. [10]

1.4 INTRODUCTION TO THE PLANTS

In our study we took four plants of genus Ocimum. They are following:

- *Ocimum sanctum* (Black variety)
- *Ocimum sanctum* (Green variety)
- *Ocimum basilicum*
- *Ocimum citriodorum*

Botanical Classification: The botanical classification for all four plants will remain same. Only species will change according to the plant.



OCIMUM SANCTUM (BLACK VARIETY)

It is also known as *Ocimum tenuiflorum*. It has two varieties. One has green leaves called as Ram Tulsi and another bears dark-purple or black leaves known as Shyam Tulsi. It has a religious use in different cultures. *Ocimum sanctum* is a strongly scented small annual herb, up to 0.5-1.5 m. tall and grows into a low bush and is commonly known as "holy basil", ‘Tulsi’ or ‘Tulasi’.

In India, holy basil is known as *tulsi*, which translates as “Incomparable one.” The plant is considered sacred. Though many herbs are revered in India, the healing powers of tulsi were recognized by the ancient rishis, giving it a special status as one of the most sacred herbs in India. It is a distant relative of the basil plant used in western cooking. In ayurveda tulsi is revered as “The elixir of life”, a great adaptogenic herb with numerous healing powers. Tulsi is worshiped by Hindus each morning and evening as the embodiment of the goddess Tulsi-devi.

Botanical name: *Ocimum sanctum*

Synonyms: Holy basil/Sacred basil/Shyam tulsi

Figure 1.1 *Ocimum sanctum* (Black)

Tulsi also known as “The Queen of Herbs" is the most sacred herb of India. Holy basil has four thousands of years been reversed and used in Ayurvedic system of medicine. *Ocimum sanctum* is little known in western world but widely cultivated in India.

Distribution:

Both forms are native to India and Southeast Asia, although they are grown around the world. Is a well known sacred plant of Indian subcontinent. It is grown almost throughout India.

Habitat:

The natural habitat of tulsi varies from sea level to an altitude of 2000 m. It is found growing naturally in moist soil nearly all over the globe. In India, Hindus grow tulsi as a religious plant in their homes, temples and their farms. They use its leaves in routine worship. This plant is also grown as a pot herb and in home gardens.

Synonyms:

Sanskrit	- Tulasi, Brinda, Manjari
Hindi	- Tulsi
English	- Holy basil / Sacred basil
Bengali	- Tulsi
Gujarati	- Tulsi
Tamil	- Thulsi
Malayalam	- Trittavu
Telugu	- Thulsi, Gaggera
Kannad	- Vishnu Tulsi
Punjabi	- Tulasi
Urdu	- Raihan

Morphological characters:

Leaves:

Leaves are green in color and posses aromatic characteristic fragrance.

They are having 2-4 cm. in length and 1-2 cm. width.

Lamina shape is oblong, ovate and is entire.

Leaf apex is acute. Venation is pinnate (parraled or straight venation).

Leaf insertion is cauline and ramal.

Leaf base is exstipulate and surface is glabrous on both side.

Stem:

Stem is green in color. It is erect unbranched and aerial. Stem posses hairs on it. Odour faintly aromatic.

Flowers:

They are small and purple to reddish color.

Seeds:

They are very small, round and somewhat flattened in shape. They are found in a group of three to four. They are shiny black in color.

Parts used:

Leaves, seeds, roots and stem

Chemical Constituents:

Leading phytoconsituents in holy basil leaf include eugenol, methyl eugenol, carvacrol, ursolic acid and rosmarinic acid. Other active ingredients are carryophylene and oleanolic acid. Seeds containd fixed oil linoleic acid, linolenic acid, stearic, palmitic and oleic acid. Nutritional components includes Vitamin A and C, minerals Ca, Iron and Zinc. It also found to contain tannins, basil camphor and flavonoids (apigenin, luteolin, orientin, vicenin),

Main medicinal and traditional uses of plant:

- Leaves are diaphoretic, antiperiodic, used in bronchitis, gastric and hepatic disorders, etc.
- A tea prepared with the leaves of *O. sanctum* is commonly used in coughs, colds, mild indigestion, diminished appetite and malaise.
- It is used as an anthelmintic, deodorant, stimulant, anti-inflammatory, cardiotonic, blood purifier, used in skin diseases and as an antipyretic particularly in malarial fevers.
- It is externally applied on chronic non healing ulcers, inflammation, skin disorders. It is useful in nausea, pain in the abdomen, worms, allergic rhinitis, all types of cough and respiratory disorders.

- *O. sanctum* leaves have been traditionally used in treatment of diabetes mellitus.
- Chewing a couple of leaves before a meal helps to stimulate the appetite, and a tea taken after a meal promotes digestion by increasing the flow of gastric juices, while reducing gas and bloating.
- In Ayurvedic medicine, the juice is recommended for snakebites, as a general tonic, and for chills, coughs, rheumatoid arthritis, anorexia, skin problems, amenorrhea and dysmenorrhea, malaria, and earaches, but mainly used in the cases of fever.
- A classic recipe advocates mixing Holy Basil, black pepper, ginger, and honey to prevent infection and to control high fevers.
- Medicinally, tulsi strengthens the immune system, increasing antibody production with its antibiotic, antiviral and antifungal properties. It is used in treating such common ailments as colds, headaches, digestive disorders, inflammation, heart disease, and various forms of poisoning and malaria.
- It is an important constituent of ayurvedic cough syrups and expectorants and in digestive remedies.
- Its properties are pungent, bitter, warming and are used in ayurveda to treat Vata and Kapha disorders. Though it does increase Pitta, it has the special power of reducing fever.
- Tulsi has been used for thousands of years to prevent and minimize the symptoms of colds and flu, to support upper respiratory health, reduce fevers and promote overall health.
- Chinese medicine uses holy basil for stomach spasms, kidney conditions, and promoting blood circulation, as well as for treating snake and insect bites.
  (A.K.Gupta 2005, Wealth of India Vol. VII, 2002, Kirtikar K.R., Basu A.R. 1984, Ayurvedic Pharmacopoeia)

OCIMUM SANCTUM (GREEN VARIETY)

Botanical name: *Ocimum sanctum*

Synonyms: Holy basil/Sacred basil/Ram tulsi

Figure 1.2 *Ocimum sanctum (*Green)

Both kind of *O.sanctum* are known as Holy basil or sacred basil but green variety is known as Ram tulsi instead of Shyam tulsi.

Distribution, Habitat, Synonyms, Chemical Constituents, uses and morphological characters all are same in both types. The major difference in morphology is the color of leaves and stem. *O.sanctum* (Green) variety has green leaves and stem instead of black or dark purple. The stem and leaves of black variety are dark purple rather than green.

OCIMUM BASILICUM

Ocimum basilicum is also known as Sweet Basil. It is a strongly scented herb having sweet aromatic fragrance. It is a culinary herb of major importance. Most culinary and ornamental basils are cultivars of the species basilicum, but other species are also grown and there are many hybrids between this species. It is a staple of Italian and Asian cooking. It is native to india although grown around the world.

It is an erect, almost glabrous herb, 30-90 cm. in height.

Botanical name: *Ocimum basilicum*

Synonyms: Sweet basil/Thai basil

Figure 1.3 *Ocimum basilicum*

Distribution:

It is distributed originally from the Middle East or the Indian subcontinent, but now cultivated throughout the tropics and subtropics. In India it is found throughout the country to an altitude of approximately 1500 m in the Himalayas, typically on or near old village sites, and very commonly on black cotton soils in the central India. It is also grown in Burma, Iran, Syria, Afghanistan, Europe, Australia, America and Africa.

Synonyms:

Sanskrit	- Surasa, Munjariki
Hindi	- Marua, Babui Tulsi
English	- Holy basil / Sacred basil
Bengali	- Babui Tulsi
Gujarati	- Damaro
Tamil	- Tirnirupachai
Malayalam	- Trittavu
Telugu	- Bhutulasi
Kannad	- Kama kasthuri
Punjabi	- Furrunj-musk, Baburi
Oriya	- Dhala Tulasi
Kashmiri	- Niazbo

Morphological characters:

Habitat:

It is indigenous to central asia and north-west India (lower hills of Punjab) and cultivated throughout India. It is grown for ornamental purpose.

Stem:

Stem is a rough, non hairy and dark puple in color. It is weak, branched and aerial.

Leaves:

The leaves are similar to basil leaves, but small and tend to be narrower.

Leaves are light green in color and posses sweet, aromatic clove like fragrance.

They are having 1.5-2.5 cm. in length and 0.5-1.5 cm. width.

Lamina shape is lanceolate and is entire.

Leaf apex is acuminate. Venation is pinate (parraled or straight venation).

Leaf insertion is cauline and ramal.

Leaf base is exstipulate and surface is glaborous on both side.

Flowers:

They are small and white or pale purple in color.

Seeds:

They are oval, subglobose and flattened in shape. They are shiny having black color. They found in a group of three. They are odourless with an oily slight pungent taste.

Parts used:

Roots, Leaves, seeds

Main medicinal and traditional uses of plant:

- *O. basilicum* is a common ingredient in Thai cuisine with a strong flavour similar to aniseed.
- It is used to flavour curries and stir-fries.
- In Chinese medicine, it is used for disturbances in renal function and gum ulcers.
- Leaf juice used for earache.
- It is also used as a hemostyptic both before and after birth.
- Sweet basil is the most popular type in North American, Italian, and other European and Mediterranean cuisines.
- It is used in teas, healing remedies, and cosmetics.

(A.K.Gupta 2005, Wealth of India Vol. VII, 2002, Kirtikar K.R., Basu A.R. 1984, Ayurvedic Pharmacopoeia)

OCIMUM CITRIODORUM

Ocimum citriodorum is also called as Lemon tulsi/Lemon basil for its lemon flavor. It is a hybrid between *O. basilicum* (Sweet basil) and *O. americanum* (African basil) and is used in cooking.

The plant bears light-green leaves and small, white flowers on stalks. Lemon basil has stems that can grow to 20-40 cm tall. Seeds form on the plant after flowering and dry on the plant.

Lemon basil is one of the most unique and delightful of the basils. One type includes a new form of lemon basil. Presently, basils which exhibit a lemon aroma and are commercially available are very short, flowering early in the summer, have small and narrow leaves, and contain a low concentration of essential oil and a low relative percent of citral in the oil. Citral is comprised of neral and geranial, chemicals that jointly cause that 'lemon' aroma.

Botanical name: *Ocimum citriodorum*

Synonyms: Lemon Tulsi/Nimbu Tulsi

Figure 1.4 Ocimum citriodorum

Distribution:

Lemon basil is a popular herb in Lao, Indonesian, Thai (Maenglak), Arabian, and Persian cuisine.

Habitat:

It is an herb grown primarily in northeastern Africa and southern Asia, for its strong fragrant lemon scent is used in cooking.

Morphological characters:

Leaves:

The leaves are similar to basil leaves, but small and tend to be narrower.

Leaves are light green in color and posses lemon like fragrance.

They are having 2.5-4.5 cm. in length and 0.5-1.0 cm. width.

Lamina shape is oval (elliptical) and entire.

Leaf apex is acute. Venation is pinnate (parraled or straight venation)

Leaf insertion is cauline and ramal.

Leaf base is exstipulate and surface is glaborous on both sides.

Stem:

Stem is a rough, non hairy and greenish in color. It is weak, branched and aerial.

Flowers:

It has white or light greenish flowers in late summer to early fall.

Seeds:

They are oval, subglobose and flattened in shape. They are shiny having brown color. They found in a group of four.

Parts used:

Leaves

Chemical Constituents:

It is found to contain volatile oil. Citral is main constituents of volatile oil which is 80% of total volatile oil. Neral and geranial are the other constituents. Linalool(4.7%), methyl eugenol, limonene, α-pinene, β-pinene also found in it. It is rich in vitamin K and beta-carotene,

Main medicinal and traditional uses of plant:

- It acts as a powerful mosquito repellent.
- The pleasant lemon fragrance of this basil makes it a great accompaniment for fish. It's also excellent in teas, potpourris, and dried arrangements.
- It is found to contains natural anti-inflammatory ingredients.
- It is an asset to a huge variety of fresh dishes, especially salads n pasta. The delicate tangy flavor adds a lovely zest to chicken, sauces, dressings, teas and soups. Lemon basil is often added to potpourri for its citrus scent.
  (A.K.Gupta 2005, Wealth of India Vol. VII, 2002, Kirtikar K.R., Basu A.R. 1984, Ayurvedic Pharmacopoeia)

2 REVIEW OF LITERATURE

21 Review of literature for *Ocimum sanctum*

2.1.1 Pharmacological Review

Antidiabetic activity: Chattopadhyay *et al* (1993) studied the anti-diabetic activity, where it was observed that oral administration of alcoholic extract of leaves of *O. sanctum* led to marked lowering of blood sugar level in normal, glucose fed hyperglycemic and streptozotocin induced diabetic rats. Further the extract potentiated the action of exogenous insulin in normal rats. The activity of the extract was 91.55 and 70.43% of that of tolbutamide in normal and diabetic rats respectively.[12]

Kar *et al* (2003) reported the comparative evaluation for hypoglycemic activity of some Indian medicinal plants in alloxan diabetic rats. *Osmium sanctum* was one of the most important plants among these. In all the experiments with different herbal samples (vacuum dried 95% ethanolic extracts), definite blood glucose lowering effect within 2 weeks have been confirmed in alloxan diabetic albino rats. Blood glucose level is brought down close to normal fasting level using herbal samples at a dose of 250 mg/kg. thrice daily.[14]

Sethi *et al* (2004) reported anti- diabetic activity in experimental animals. Dietary supplementation of fresh tulsi leaves in a dose of 2 gm/kg body weight for 30 days led to significant lowering of blood glucose levels in test group. Intake of *Ocimum sanctum* also led to significant increase in levels of superoxide dismutase, reduced glutathione and total thiols, but marked reduction in peroxiodised lipid levels as compared to untreated control group. The leaves were found to possess both superoxide and hydroxyl free radical scavenging action. The present observations establish the efficacy of *Ocimum sanctum* leaves in lowering blood glucose levels and antioxidant property appears to be predominantly responsible for hypoglycemic effect.[13]

Khan *et al* (2010) investigated antidiabetic effects of ethanolic extract of the leaves of *Ocimum sanctum* in normal and alloxan induced diabetic rats (AIDRs). The effect of the extract (200 mg/kg body weight i.p) on fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), serum glutamate oxaloacetate transaminases, serum

glutamate pyruvate transaminases (SGOT, SGPT) level, and liver glycogen content were investigated and found significant effect. In diabetic rats, SGOT and SGPT levels were significantly elevated that were further reduced after intraperitoneal administration of the extract. These results indicate that ethanolic extract of the leaves of *O. sanctum* have favorable effects in bringing down the severity of diabetes together with hepatoprotectivity.[11]

Analgesic and Antipyretic activity-- Godhwani *et al* (1997) reported that the methanolic extract and an aqueous suspension of *Ocimum sanctum* inhibited acute as well as chronic inflammation in rats as tested by carrageenan-induced pedal edema and croton oil-induced granuloma and exudates, respectively. In both test procedures, the anti-inflammatory response of 500 mg/kg of methanolic extract and aqueous suspension was comparable to the response observed with 300 mg/kg of sodium salicylate. Both the extract and suspension showed analgesic activity in the mouse hotplate procedure and the methanolic extract caused an increase in the tail-withdrawal reaction time of a subanalgesic dose of morphine. Both preparations reduced typhoid-paratyphoid A/B vaccine-induced pyrexia.[15]

Antiinflammatory activity- Singh et al (1998) reported anti-inflammatory activity of the fixed oil containing α-linolenic acid obtained from *Ocimum sanctum.* It was screened for their anti-inflammatory activity using carragenan, leukotriene and arachidonic acid induced paw edema models in rats and the antiinflamatory effects were compared with the standard drug indomethacin. It was found [16]

Antimicrobial activity: Mahmood *et al* (2008) reported that the ethanol extract of *O.sanctum* showed antibacterial activity, greater in Gram positive bacteria than gram-negative, especially against *B.subtilis* and *S.aureus*; comparatively less than *Origanum majorana*. Another study on *O.sanctum* essential oil showed marked antibacterial efficiency against all bacteria tested, like *E. coli*, *Klebsiella* sp., *P. mirabulus*, *P. aeruginosa* and *S. aureus*. by disc-diffusion method. The essential oil exhibited significant antibacterial activity against all the test pathogens, with maximum zone of inhibition against *S. aureus* (20.0 mm & 41.5 mm) and minimum against *E. coli* (10.2 mm & 17.8 mm) for 5 and 10 µl of essential oil, respectively.[17]

Antifungal Activity: Taylor *et al* (1995) studied aqueous extracts and oils of five Indian medicinal plants, traditionally used for their antimicrobial activities. Extract and oils were evaluated against two of the most prevalent Candida species causing candidiasis, *C. albicans* and *C. tropicalis*. Of these plant materials, three showed varying degrees of antifungal activity against both species. Tulsi essential oil was found to be the most effective. Eugenol, methyl eugenol, linalool, and 1, 8-cineole, along with tulsi essential oil were then evaluated at the same.[18]

Kumar *et al* (2010) studied the efficacy of *Ocimum sanctum* essential oil and its major component, eugenol against the fungi causing biodeterioration of food stuffs during storage. *O. sanctum* essential oil and eugenol were found efficacious in checking growth of *Aspergillus flavus* and their minimum inhibitory concentrations were recorded as 0.3 and 0.2 µl respectively.Both of these were found superior over some prevalent synthetic antifungal and exhibited broad fungitoxic spectrum against 12 commonly occurring fungi. The findings of present study reveals the possible exploitation of *O. sanctum* essential oil and eugenol as plant based safe preservatives against fungal spoilage of food stuffs during storage.[19]

Larvicidal Activity: Anees M *et al* (2008) reported the larvicidal activity of *O.sanctum*. The acetone, chloroform, ethyl acetate, hexane, and methanol leaf and flower extracts of *O. sanctum* were studied against fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*. The highest larval mortality was found in leaf extract of *O. sanctum* against the larvae of *A. aegypti* and *C. quinquefasciatus*.[20]

Immnostimulatory Activity: Logambat *et al* (2000) investigated the immunostimulatory effect of *O.sanctum.* Effect of leaf extract of *Ocimum sanctum* on the specific and non-specific immune responses and disease resistance against *Aeromonas hydrophila* was investigated in *Oreochromis mossambicus*. Leaf extract of *O. sanctum*, when administered intraperitoneally, stimulated both antibody response and neutrophil activity. Dietary intake of *O. sanctum* also enhanced the antibody response and disease resistance against *A. hydrophila*. Possibility of using *O. sanctum* as immunostimulant in the maintenance of finfish health in intensive freshwater aquaculture is suggested.[21]

Antioxidant activity: Sethi *et al* (2004) showed the leaves of *O.sanctum* to possess both superoxide and hydroxyl free radical scavenging effect and attributes the antioxidant property to be responsible for its hypoglycemic effect.[13]

Kath *et al* 2006 reported that the hydroalcoholic extract of *O.sanctum* leaves has been investigated for its antioxidant activity in animal models of peptic ulcer with the aim of exploring a possible correlation between its antioxidant and antiulcer activities. Gastric ulcers were produced in rats by ethanol treatment and pyloric ligation whereas duodenal ulcers were produced in guinea pigs by histamine treatment. The antioxidant activity was evaluated estimating plasma in ethanol treated rats and histamine treated guinea pigs.[22]

Cardioprotecdtive effect: Sharma *et al* (2001) study showed *Ocimum sanctum* has cardioprotecdtive effects against isoproterenol induced myocardial necrosis probably through improved ventricular function, augmentation of endogenous antioxidants and suppression of oxidative stress. [23]

Anti-cancer activity: Karthikeyan *et al* (1999) investigated the anti-cancer activity against human fibrosarcoma cells (HFS cells) in culture. Administration of aqueous and ethanolic extracts of *Ocimum sanctum* to mice bearing sarcoma solid tumors mediated a significant reduction in tumor volume and an increase in lifespan. These observations clearly indicate *O.sanctum* extracts possess anticancer activity[24]

Anti-Ulcer activity: Mandal *et al* (1993)l reported that the extract of *Ocimum sanctum* possess antiulcerogenic properties with reduction of the ulcer index, free and total acidity in rats. Seven days of treatment increased mucous secretion.[25]

Hepatoprotective activity: Gupta *et al* (2006) reported that the leaf extract of *O.sanctum* have a hepatoprotective effect on hepatotoxicty induced by antitubercular drugs. The exact mechanism has not been defined, but *O.sanctum* antioxidant activity seems to be the most important mode of its hepatoprotective effect.[26]

Anti-Noise/Stress Alleviating effect: Bhargva *et al* (1981) reported that the ethanolic extract on noise stress induced changes in albino rats leucopenia, increased corticosterone levels and enhanced neutrophil functions as indicated by increase in

Candida phagocytosis showed normalization of the altered values by pretreatment with *O.sanctum* extract.[27]

Radioprotective effect: Devi *et al* (1998) studied the radioprotective effects of two flavonoids, orientin and vicenin from the leaves of *O.sanctum* by evaluating chromosome aberration in bone marrow cells of irradiated mice. Flavonoids may be promising for human radiation protection.[28]

CNS-Protective activity : Sakina *at al* (1990) reported CNS protective effect of ethanol leaf extract of *O.sanctum* against haloperidol-induced catalepsy. Results indicates that *O.sanctum* may be used to prevent drug-induced extrapyramidal effects.[29]

Anti-stress activity: Bhargva *et al* (1981) studied the anti-stress activity in *O.sanctum.* Ethanolic extract study showed leaves possess significant anti-stress effects probably through a central nervous system pathway that may involve the GABA-ergic system. Another study on noise-induced changes in rats were normalized with pretreatment with *O.sanctum* extract indicating its stress-alleviating effect.[27]

Anti-tussive effect: Pratibha *et al* (2005) reported the antitussive effect. Study shows an antitussive effect probably by central action mediated through both opioid and GABA-ergic system. The experiment was carried out on healthy male guinea pigs, where cough was induced by exposure to aerosol of citric acid. It was introduced through a small opening at the sides of the chamber using an ultrasonic nebulizer for 5 minutes. The observation after a certain time shows the significant anti-tussive effect by aqueous and methonolic extract of *O.sanctum*.[30]

Anthelmintic Activity: Asha *et al* (2001) reported that the essential oil of *Ocimum sanctum* and its chief constituent eugenol. Their study showed potent in vitro anthelmintic activity in the caenorhabditis elegant model. Eugenol exhibited an ED_{50} of 62.1 µg/ml. Eugenol being the predominant component of the essential oil, is suggested as the putative anthelmintic principle.[31]

Antihyperlipidemic Activity: Iyer *et al* (1997) reported the effect of *Ocimum sanctum* leaf powder supplementation on blood sugar levels, serum lipids and tissue lipids in diabetic rats. They fed Tulasi leaf powder at 1% level in normal and diabetic rats for a

period of one month to explore the effect on fasting blood sugar, uronic acid, total amino acids, and the lipid profile in serum and tissue lipids. The results indicated a significant reduction in fasting blood sugar, uronic acid, total amino acids, total cholesterol, triglyceride, phospholipids and total lipids. In liver, total cholesterol, triglyceride and total lipids were significantly lowered. Total lipids were significantly reduced in kidney. In heart, a significant fall in total cholesterol and phospholipids was observed. All these observations indicate the hypoglycemic and hypolipidemic effect of Tulasi in diabetic rats.[32]

2.1.2 Chemotaxonomical Review

Leopold *et al* (2003) analyzed the essential oil aroma compounds of four different Ocimum species (*O.americanum*, *O.basilicum*, *O.gratissimum* and *O.sanctum*) from southern India by solid phase microextraction (SPME)/gas chromatography (GC)/flame ionization detection (FID), SPME/GC/mass spectrometry (MS) and olfactory evaluations. The essential oil of leaf of *O. basilicum* contains (E)-methyl cinnamate (34.49%), linalool (28.44%), camphor (13.08%), (Z)-methyl cinnamate (6.90%) and geraniol (3.84%), whereas the essential leaf oil of *O. gratissimum* comprises eugenol (63.36%), (Z)-bocimene (9.11%), germacrene D (8.84%) and bcaryophyllene (3.89%). The essential leaf oil of *O.sanctum* shows methyl eugenol (56.18%), caryophyllene (16.60%) and germacrene (5.10%) as main constituents. Therefore, the following chemotypes can be attributed to the analyzed Ocimum samples: *O.americanum*, methyl cinnamate-type; *O.basilicum*, methyl cinnamate/linalool-type; *O.gratissimum*, eugenol-type; and *O.sanctum*, methyl eugenol-type. They identify the aroma compounds of the essential oils of these four Ocimum species from Kerala responsible for the characteristic odor of the single sample, to find out the chemotype of each and to discuss a possible use for them in the flavouring of food products. [8]

Angers *et al* (1996) determined the oil content, fatty acid composition and glyceride profile of oil from seeds of seven Ocimum species. The species were included *O.basilicum, O.canum, O. gratissimum*, and *O.sanctum*. The oil content ranged from 18 to 26%, with triglycerides comprising between 94 and 98% of extracted neutral lipids. The major acylated fatty acids were linolenic (43.8–64.8%), linoleic (17.8–31.3%), oleic (8.5–13.3%), and palmitic acid (6.1–11.0%). Linolenic acid was similar among

the four *O. basilicum* chemotypes (57–62%), highest in *O.canum* (65%), and lowest in *O.sanctum* (44%). Basil seed oil appears suitable as an edible oil or can be used for industrial purposes, and could be processed in the same way as linseed oil.[33]

2.1.3 Chemical characterization Review

Roberto *et al* (1998) isolated essential oils from Ocimum species by steam distillation and the composition of volatile oil constituents was used to characterize the diversity among the most economically important Ocimum species. Using principal component analysis on the aromatic volatile oils, the Ocimum accessions could be separated into five groups, which do not correspond to the different species: (1) citral-spathulenol accessions; (2) linalool-rich accessions; (3) methylchavicol-rich accessions; (4) linalool-methylchavicol accessions; and (5) methyl(*E*)-cinnamate-rich accessions. The study revealed that the groups of Ocimum species are based on morphological characteristics does not correspond to the groups based on volatile oil constituents.[34]

2.1.4 Phytochemical Review:

Hakkim *et al* (2007) studied, the chemical constituents and antioxidant property of *Ocimum sanctum*. Field-grown plant parts (leaves, stems, and inflorescence) were compared with those of respective callus cultures induced from each explant in *in vitro*. The callus cultures were successfully initiated on MS medium supplemented with 2,4-dichlorophenoxy acetic acid combined with different concentrations (0.1-0.5 mg/L) of kinetin as plant growth regulators. The distribution of phenolic compounds in these extracts was analyzed using reverse phase high-performance liquid chromatography with reference standards. Interestingly, rosmarinic acid was found to be the predominant phenolic acid in all callus extracts.[35]

Himal *et al* (2008) performed a qualitative phytochemical analysis for the detection of phytoconstituents in *Ocimum sanctum*. They reported the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugar in the ethanolic extract.[36]

2.2 Review of literature for *Ocimum basilicum*

2.2.1 Pharmacological Review

Antimicrobial Activity: Ahmet *et al* (2005) investigated in-vitro antimicrobial properties for ethanol, methanol, and hexane extracts from *Ocimum basilicum.* A total of 146 microbial organisms belonging to 55 bacteria, and four fungi, and a yeast species were studied using a disk-diffusion and minimal inhibition concentration method. The result showed that none of the three extracts tested have antifungal activities, but anticandidal and antibacterial effects were found significant. Both the hexane and methanol extracts, but not the ethanol extracts, inhibited three isolates out of 23 strains of *Candida albicans* studied. All three extract of *O. basilicum* were different in terms of their antibacterial activities.[38]

Almeida *et al* (2007) reported the effect of *Ocimum basilicum* essential oil on *Giardia lamblia* and on the modulation of the interaction of these parasites by peritoneal mouse macrophage. The essential oil (2 mg/ml) and its purified substances demonstrated antigiardial activity. Linalool (300 µ/ml) was able to kill 100% parasites after 1 h of incubation, which demonstrates its high antigiardial potential. These results suggest that with *Giardia lamblia,* the essential oil from *Ocimum basilicum* have a potent antimicrobial activity.[39]

Kaya *et al* (2008) studied the antimicrobial activities of chloroform, acetone and two different concentrations of methanol extracts of *Ocimum basilicum* L. These extracts were tested *in vitro* against 10 bacteria and 4 yeasts strains by the disc diffusion method. The results indicated that the methanol extracts of *O. basilucum* exhibited the antimicrobial activity against tested microorganisms. While the chloroform and acetone extracts had no effect, the methanol extracts showed zone of inhibition against strains of *Pseudomonas aeruginosa, Shigella sp., Listeria monocytogenes, Staphylococcus aureus* and two different strains of *Escherichia coli*.[37]

Gupta *et al* (2009) reported the antibacterial activity in *O.basilicum*. The study was designed where aerial parts of *Ocimum basilicum* were extracted with organic solvents (ethanol, methanol, ethyl acetate and hexane) and then evaluated for *in vitro* activity by using agar well diffusion method against *Staphylococcus aureus, Bacillus subtilis,*

Bacillus cereus, Pseudomonas aeruginosa, Escherischia coli, and *Salmonella typhi*. The methanol extract showed prominent effect on the tested microorganisms. However, *Salmonella typhi* was found to be most resistant against the extracts.[40]

Anti-Ulcer activity: Akhtar *et al* (1989) were studied antiulcerogenic activities of *O.basilicum* against aspirin-induced gastric ulcers in rats. In addition, their effects on output of gastric acid and pepsin and hexosamine concentrations in gastric fluid were recorded in ulcerated and non-ulcerated rats. *O.basilicum* (aerial parts) powder and its aqueous and methanolic extracts decreased the index. Moreover, the acid output was decreased by its methanolic extract while hexosamine secretion was enhanced. This suggests that its antiulcerogenic effect is due to decreases of acid and pepsin outputs which enhance gastric mucosal strength. The reference drug gefarnate decreased the ulcer index by increasing the hexosamine level only.[41]

Anti-hyperglycemic and hypolipidemic activity: Zeggwagh *et al* (2007) reported the hypoglycemic and hypolipidemic effects of the aqueous extract of *Ocimum basilicum.* After a single oral administration of aqueous extract of *Ocimum basilicum* significantly reduced blood glucose levels in normal and diabetic rats. After 15 days of repeated oral administration, it produced a potent reduction on blood glucose levels in diabetic rats and a less reduction in normal rats. Total plasma cholesterol and triglycerides levels were significantly reduced after repeated oral administration in diabetic rats. However, no change was observed in total plasma cholesterol and triglycerides levels in normal rats after both single and repeated oral administration.[42]

Hepatoprotective activity: Amani *et al* (2009) reported hepatoprotective activity. Six triterpene acids betulinic, oleanolic, ursolic, 3-epimaslinic, alphitolic and euscaphic acids have been isolated from a dichloromethane extract of hairy root cultures of *Ocimum basilicum.* These cultures were obtained by genetic transformation using *Agrobacterium rhizogenes.* The extracts as well as the isolated compounds were evaluated for their hepatoprotective activity by measuring their effect on the oxidative stress status of liver, in albino rats and in liver homogenate *in vitro*. All tested compounds displayed hepatoprotective activity comparable to oleanolic and ursolic acids.[43]

Antioxidant activity: Meera *et al* (2009) studied the significant antioxidant activity were obtained by ethanolic extract of leaves of *O.basilicum* against liver damage induced by H_2O_2 and CCl_4 as evidenced by decreased levels of antioxidant enzymes. The extract also showed significant antilipid peroxidation effects *in vitro*, besides exhibiting significant activity in superoxide radical and nitric oxide radical scavenging, indicating the potent antioxidant effects.[44]

2.2.2 Chemical characterization review

Hussain *et al* (2008) investigated the essential oils from aerial parts of *Ocimum basilicum L.* as affected by four seasonal, namely summer, autumn, winter and spring growing variation. The hydro-distilled essential oils content ranged from 0.5% to 0.8%, the maximum amounts were observed in winter while minimum in summer. The essential oils consisted of linalool as the most abundant component 60.6%, followed by cadinol 11.4%, bergamotene 9.2% and cadinene 5.4%. Samples collected in winter were found to be richer in oxygenated monoterpenes (68.9%), while those of summer were higher in sesquiterpene hydrocarbons (24.3%). The contents of most of the chemical constituents varied significantly with different seasons.[45]

2.2.3 Phytochemical Review:

Tateo *et al* (1989) reported that *Ocimum basilicum* L. contains eugenol which is responsible for its clove scents. The dried leaves of *O.basilicum* contain 0.20-1% essential oils. Literature revealed that about 45 compounds are found in volatile oils of this plant with the major compounds being linalool, eugenol, methylchaviol, methylcinnamol, linolen, olimene, pinene, cineol, anethol, estragol, thymol, citral and camphor.[47]

Wossa *et al* (2008) studied the volatile chemical constituents of three ocimum species. Fresh aerial parts of three species of basil, *Ocimum basilicum*, *O. tacilium* and *O. canum* were subjected to exhaustive hydrodistillation to afford pale yellow colored oils in 1.0, 0.7 and 0.01 percent yields respectively. Detailed chemical evaluation by GC and GC/MS revealed *O. basilicum* to be composed of a total of eleven components representing 100 percent of the total oil composition. Neral (36.1 %) and geranial (44.5 %) were found to be the major components.[48]

Sanni *et al* (2009) reported the presence of saponins and alkaloids in high amount while flavonoids, terpenes and steroids in medium quantity and traces of tannins and carbohydrates in the aqueous extract of leaves of *O.basilicum*. The phytochemical investigation of the various solvent fractions showed that all the three fractions (chloroform, n-butanol and ethyl acetate fractions) contain carbohydrates, terpenes and steroids, tannins and flavonoids.[46]

2.3 Review of literature for *Ocimum citriodorum*

Hakkim *et al* (2008) reported antioxidative properties of eight selected Ocimum species (*Ocimum gratissimum, Ocimum americanum, Ocimum minimum, Ocimum citriodorum, Ocimum kilimandscharicum, Ocimum grandiflorum, Ocimum lamiifolium, and Ocimum selloi*). Leaves of these plants were extracted using methanol. The quantitative analysis of phenolic constituents was determined using high-performance liquid chromatography. Total phenolic content was estimated using Folin-Ciocalteu reagent and antioxidant activity was assessed using iron (III) reduction, carotene, linoleic acid-bleaching, 1,1-diphenyl-2-picrylhydrazyl and superoxide anion free radical scavenging assays. Phenolic acids, hydroxycinnamates, and flavonoids were identified and quantified within each extracts based on the area of each peaks with an external standards. The extracts of Ocimum species exhibited activity in all the in-vitro antioxidant assays but it was not as potent as butylated hydroxyl anisole. The phytochemicals found in each extract are rich antioxidants and these extracts can be used as an effective preservative in food industry.[49]

4. EXPERIMENTAL WORK

Identification, collection and authentication of samples

Fresh leaves were collected from Nirma Herbal Garden, Institute of pharmacy, Nirma University, Ahmedabad.

Authentication was done by ethanobotanist Dr. Bhaskar Punjani, Gandhinagar by studying the mega morphological features.

Preparation of samples

The plant material (leaves) was washed thoroughly with distilled water, pressed between folds of filter paper and dried at room temp. for 2-3 days. They were then dried in hot air oven at 50°C for 1 hr. The dried leaves were then ground to fine powder to 40 mesh size. Powdered material was stored in airtight glass container at 10°C till further use. The powder material was used for screening of various parameters like phytochemical, physiochemical parameters etc.

4.1 Morpholgical study

The fresh leaves of all four species of genus Ocimum collected from herbal garden were studied for the morphology and compared with characters mentioned in the literature. (The Ayurvedic Pharmacopoeia of India) [50]

4.2 Microscopical study

For microscopic evaluation various free hand transverse sections of fresh leaves of all four species were taken. Then unstained slides were prepared and examined under microscope and photographs were scanned with CCD camera.

Microscopy of powder was also carried out. For this powder samples were boiled with chloral hydrate in a test tube and both unstained and stained slides were prepared and examined under microscope.

Quantitative analysis of leaves of all four species were done by measuring various leaf constants like stomatal number, stomatal index, palisade ratio, vein islet number and vein let termination number.

Procedure:

First of all we clear the piece of the leaf by boiling with chloral hydrate solution. Then peeled out upper and lower epidermis separately by means of forcep. It was keep on slide and mount in glycerin. Further it was subjected for evaluation of various leaf constants and diagrams were scanned.[51]

4.3 Phytochemical analysis[51]

Various extracts (petroleum ether, chloroform, ethyl acetate and methanol) prepared separately from the powder of various species of Ocimum. Then they were subjected to the following tests separately for the detection of presence of various phytoconstituents viz. alkaloids, flavonoids, saponins, carbohydrates, steroids, terpenoids, glycosides, tannins and phenolic compounds.

Test for alkaloids:

Evaporate the aqueous, alcoholic and chloroform separately. To residue, add dilute HCl. Shake well and filter. Filtrate was using to perform following tests.

(a) Mayer's test: To a few ml. of filtrate, a drop of Mayer's reagent (mercury potassium iodide) was added by the side of the test tube and observed. A white or creamy precipitate indicates the presence of alkaloid. (Evans, 1997)

(b) Wagner's test: To a few ml. of filtrate, few drops of Wagner's reagent (iodine reagent) was added by the side of the test tube and observed. A reddish brown precipitate indicates the presence of alkaloid. (Wagner, 1993)

(c) Hager's test: To a few ml of filtrate, 1 ml of Hager's reagent (Saturated solution of picric acid) was added and observed. Prominent yellow precipitate indicates the presence of alkaloid.

(d) Dragendorff's test: To a few ml of filtrate, 1 ml of Dragendorff's reagent (potassium bismuth iodide) was added and observed. A prominent yellow precipitate indicates the presence of alkaloid. (Wagner et al, 1996)

Test for carbohydrates:

(a) Molisch's test:

To 2 ml of aqueous extract, two drops of alcoholic solution of α-naphthol was added. The mixture was shaken well and 1 ml of concentrated sulfuric acid was added slowly along the side of the test tube and allowed to stand. A violet colored ring indicates the presence of carbohydrate.

(b) Fehling test:

1 ml of aqueous filtrate was boiled on water bath with 1 ml each of Fehling solution A (copper sulphate) and B (sodium potassium tartarate) and observed. A red precipitate indicates the presence of sugar.

(c) Barfoed's test:

To 1 ml of filtrate, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes and observed. Red precipitate indicates presence of sugar.

(d) Benedict's test:

To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. and observed. A characteristic colored precipitate indicates the presence of sugar.

Test for glycosides:

(a) Borntrager's test:

Powder of drug was extracted with ether. To the filtered ethereal extract 10% ammonia solution was added and observed. Pink color indicates the presence of anthraquinone glycosides. (Evans, 1997)

(b) Legal's test:

To aqueous or alcoholic extract add 1 ml. pyridine and 1ml. sodium nitroprusside solution. Pink color indicates the presence of glycosides. (Raaman, 2006)

Tests for Saponins:

(a) Foam test:

Shake the drug extract or dry powder vigorously with water. Persistent foam observed which indicates the presence of saponins. (Kokate , 1999)

(b) Haemolytic Test:

Add drug extract or dry powder to one drop of blood placed on glass slide. Haemolytic zone appears which indicates the presence of saponins. (Kokate, 1999)

Tests for Sterols and Triterpenoids:

(a)Lieberman Buchardt test:

1 gm. Powdered drug was moistened with 1 ml. of acetic anhydride and 2-3 drops of concentrated sulphuric acid on a clean tile. The powder was mixed well and the color gained was observed. Purple to violet color indicated the presence of triterpenoids. (Finar, 1986)

(b)Salkowski reaction:

To 2 ml. of extract, 2 ml. chloroform and 2 ml. conc. sulphuric acid were added and shake well. Chloroform layer appeared red and acid layer gives greenish yellow color.

Test for Flavonoids:

(a) Shinoda test:

1 gm. of leaves powder was extracted with 10 ml. of ethanol (95 %v/v) for 15 min. on water bath and filtered. To the filtrate few pieces of magnesium ribbon were added. To this, 3 to 4 drops of concentrated hydrochloric acid was added and observed. Pink to crimson color indicates the presence of flavanoids. (Harborne, 1998)

(b) Fluorescence test

1 gm. powder was extracted with 15 ml. methanol for 2 min. on boiling water bath, filtered while hot and evaporates to dryness. To the residue 0.3 ml. boric acid (3% w/v) and 1 ml. oxalic acid solution (10% w/v) were added. The mixture was evaporated to dryness and the residue was dissolved in 10 ml. ether. The ethereal layer shows fluorescence under UV light indicates presence of flavonoid. (Geissman A., 1955)

Tests for Tannins:

Aqueous extract of the leaves powder was prepared by refluxing 10 gm. of powder with 50 ml of water for about 1 h on water bath and was used for the following tests;

(a) Gelatin test:

To 2-3 ml. of aqueous extract, add 1% gelatin solution containing 10% NaCl and observed. White precipitate indicates the presence of tannins. (Raaman, 2006)

(b) Lead acetate test:

To 2 ml. of aqueous extract of drug, 3 ml of 10 % lead acetate solution was added and observed. The bulky white precipitate indicates presence of tannins.(Raaman, 2006)

Tests for Phenolic compounds:

(a) Ferric chloride test:

Methanolic extract of powdered leaves was taken. To this a drop of freshly prepared ferric chloride solution (5%) was added. Dark green color indicates the presence of phenolic compounds. (Raaman, 2006)

(b) **Test with Folin ciocalteu reagent:**

To a drop of methanolic extract of leaves powder, a drop of Folin ciocalteu reagent was added and observed. Bluish green color indicates the presence of phenolic compounds. . (Feigle, 1956b)

4.4 Physiochemical analysis[50, 52]

Determination of ash value:

Ash values of powder of leaves of four varieties of genus Ocimum were determined by the following method:

(a) Determination of total ash:

About 4 g of the ground air-dried powdered material was accurately weighed, in a previously ignited and tarred silica crucible. The material was spread in an even layer and ignited by gradually increasing the heat to 500-600°C until it was white, indicating the absence of carbon. It was allowed to cool in a desiccator and weighed. The percentage content of total ash was calculated with reference to air-dried material.

(b) Determination of acid insoluble ash:

In crucible containing the total ash, 25ml of hydrochloric acid (2N) was added, crucible was covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot distilled water and this liquid was added to the crucible. The insoluble matter on an ash less filter-paper was washed with hot water until the filtrate was neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hotplate and ignited to constant weight. Allowed the residue to cool in a suitable desiccator for 30 minutes, and then weigh. The percentage content of acid insoluble ash was calculated with reference to air-dried material.

(c) Determination of water soluble ash

In crucible containing the total ash, 25ml of water was added and boiled gently for 5 minutes. Insoluble matter was collected on ash less filter paper and washed with hot

water until the filtrate was neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hotplate and ignited in a crucible to constant weight, at a temperature not exceeding 450°C. Allowed the residue to cool in a suitable desiccator for 30 minutes, and then weigh. Weight of the residue was subtracted from weight of total ash. The percentage content of water soluble ash was calculated with reference to air-dried material.

Determination of Extractive value:

(a) Determination of water soluble extractive value:

About 4 g of the air-dried drug was macerated with 100 ml of water in a closed flask, shaken frequently during 6 hours and allowed to stand for 18 hours. The solution was filtered, 25 ml of the filtrate was evaporated to dryness in a petri-dish on water-bath, and dried at 105 ^{o}C, cooled in a desiccator for 30 minutes and weighed without delay. Percentage water-soluble extractive value was calculated with reference to the air-dried drug.

(b) Determination of alcohol soluble extractive value:

About 4 g of coarsely powdered air-dried material was macerated in a glass-stoppered conical flask with 100 ml of the methanol, shaken frequently during 6 hours, allowed to stand for 18 hours. Filtered rapidly, care was taken not to lose any solvent; 25 ml of the filtrate was transferred to a petri-dish and evaporated to dryness on a water-bath. Extract was dried at 105 °C, cooled in a desiccator for 30 minutes and weighed without delay. Percentage alcohol soluble extractive value was calculated with reference to the air-dried drug.

Determination of loss on drying

About 5 gm. of air dried plant material was weighed in Petri-dish, dried in oven at 100-105ºC until two consecutive weighing was not differ by more than 5 mg. Percentage loss of weight was calculated with reference to the air-dried drug.

4.5 Method for fluorescence analysis [53, 54]

Many phyto drugs when suitably illuminated emit light of different wave length or color from that which falls on them the fluorescence analysis of drug helps to identify the drug with specific fluorescent colors and also to find out the fluorescent impurities. The study of fluorescence analysis can be used as a diagnostic tool for testing adulteration. Fluorescence analysis carried out by treating powder with various chemicals and reagents like Conc. HCl, Conc. H_2SO_4, Nitric acid etc. and then observed under visible and U.V. light.

4.6 Isolation of essential oil[51]

Plant material

Fresh leaves of all four species of genus Ocimum were collected from the Nirma Herbal Garden, Institute of Pharmacy, Nirma University, Ahmedabad. Leaves were washed under tap water and cut into small pieces to expose the oil sacs. Fragrant essential oil was extracted from these crushed leaves by steam distillation.

Isolation of essential oil

Fifty gram of fresh leaves of Ocimum species were subjected to steam distillation separately in a clevenger type apparatus for three hours. Two layers were formed, upper organic layer of oil and lower aqueous layer of water. Lower aqueous layer was discarded and upper layer of oil was collected in apendorf tubes. Light yellow oil was further refined with Na_2SO_4 in order to remove traces of water if present. The essential oil sample (0.1 ml.) was stored in dark brown bottle and was kept in refrigerator. This process was repeated six times to get sufficient amount of oil to perform TLC and antimicrobial assay. Yield of essential oil obtained having sharp essence was vary with different Ocimum species. The result was expressed as a volume in weight percentage.

TLC profile of Volatile Oil: Chromatographic separation for all volatile oil obtained from four different species was done on precoated TLC Silica gel 60 F_{254} plates (Merck). The sample was prepared by dissolving the volatile oil in toluene in ratio of 1:10 (0.1 ml. volatile oil was diluted up to 1ml. of toluene). The samples were applied on TLC plate and the plate was developed in a solvent system of toluene: ethyl acetate: (93:7) in a TLC chamber. After drying the TLC plate at room temp. spots and bands

were visualized by UV irradiation (254 and 366 nm) and also after derivatization with Vanillin - Sulphuric acid as spraying reagent.

4.7 HPTLC finger printing[55]

Materials and Methods

Reagents and Chemicals

- Toluene (LR Grade, S.D. Fine chemicals Ltd., Mumbai, India)
- Ethyl acetate (LR Grade, S.D. Fine chemicals Ltd., Mumbai, India)

HPTLC Conditions

- Mobile phase: Toluene : Ethyl Acetate (97: 3, v/v)
- Wavelength of detection: 254nm and 366nm

Preparation of Stock Solution of Oil:

0.1ml of oil obtained from leaves of various Ocimum species was diluted up to 1 ml with toluene. These solutions were utilized for HPTLC fingerprinting.

HPTLC Analysis

HPTLC finger print profile was established for volatile oil of all four species using HPTLC. It was developed to identify and assure the presence of various constituents in the volatile oil. All volatile oils were diluted separately with toluene in ratio of 1:10 (0.1 ml. of volatile oil diluted up to 1 ml. with toluene). Now this suitably diluted oil was spotted on pre-coated Silica gel G60 F254 TLC plates and the plates were developed in solvent system toluene: ethyl acetate (93:7). The plates were dried at room temperature and scanned using TLC Scanner 3 (CAMAG) at 254 nm (absorbance/reflectance mode) and 366 nm (fluorescence/reflectance mode) and R_f values, spectra, λ_{max} and peak area of the resolved bands were recorded. Relative percentage area of each of the band was calculated from peak areas.

4.8 Antibacterial activity[56,57,58]

The aim of present study was to screen the comparative antibacterial efficacy of essential oils isolated from various Ocimum species against both gram-positive (*S.aureous*) and gram-negative strains (*E.coli*).

Protocol for antimicrobial activity

(a) Source of microorganism:

Two bacterial strains (Gram positive: *Staphylococcus aureus* ATCC-29737, *and* Gram-negative: *Escherichia coli* ATCC-10536) were selected from the microorganisms given in United States Pharmacopoeia (2000), British Pharmacopoeia (1993) and Indian Pharmacopoeia (1996) for antimicrobial assays. The organisms were procured from American Type Culture Collection (ATCC), B. V. Patel Pharmaceutical Education and Research development Centre, Thaltej, Ahmedabad, India.

(b) Standardization of microorganism:

Exactly 0.2 ml of overnight culture of each organism were dispensed into 20ml of sterile nutrient broth and incubated for 3-5 hour to standardize the culture to 10^6 cfu/ml. 1 ml of the standardized culture was used for the anti microbial activity.

(c) Isolation of volatile oil from leaves:

Volatile oils were isolated from all four species by using method mention earlier.

(d) Preparation of various concentrations of test oil:

5 and 10 µl. of undiluted essential oil was used as test oil. The reference standard of ofloxacin was prepared using market preparation, ofloxacin Injection I.P., (QUINTOR, 200mg/100ml). Serial dilutions were made with water for injection (WFI) (Nirlife Healthcare) as diluent.

(e) Optimization of DMSO concentration:

Different concentrations of DMSO were used for the optimization (1%, 2%, 3%, 4%, 5% v/v). Microbial suspensions were added to the Petri plates containing agar (2.8%, 25ml) and incubated at 37^{o}C. The Petri plates were observed for the growth of the microorganisms for 24 h.

(f) Preparation of culture media:

Nutrient agar media was used for the preparation of the stock cultures and sub-cultures of all bacteria used for the pharmacological evaluation of test oil. Nutrient Agar media (M00-500G) was purchased from HI MEDIA Laboratories Ltd., Mumbai, India. Freshly prepared Nutrient Agar media was used as per requirement for pharmacological activity of test extract. Composition and preparation of the Nutrient Agar is mentioned below.

Agar medium was prepared according to direction provided by manufacturer. 31.55 g of nutrient agar was suspended in 1000 ml distilled water. It was heated to boiling to dissolve the medium completely. It was sterilized by autoclaving at 15lbs pressure (121^{0}C) for 15 minutes. It was then cooled to 45 ^{0}C, mixed thoroughly and poured into sterile petriplates.

Composition of Nutrient Agar media:

Sr. No.	Ingredients	Quantity (g/litre)
1	Peptic digest of animal tissue	5.0
2	Sodium chloride (NaCl)	5.0
3	Beef extract	1.5
4	Yeast extract	1.5
5	Agar	15.0

* Final pH (at 25°C) 7.5 $\pm$ 0.2

Petriplates were incubated for 24 hr at 37^{o}C.The results were recorded by measuring the diameter of inhibition zone at the end of 24-48 h.

4.9 Antifungal Activity[56,59,60]

Experimental protocol

In present work, the volatile oils were evaluated for *in vitro* antifungal activity using agar well diffusion methodology against several fungal strains using Rose Bengal agar (Himedia).

Procedure

(A) Preparation of media

Agar medium was prepared according to direction provided by manufacturer. 31.55 g of rose bengal agar was suspended in 1000 ml distilled water. It was heated to boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. It was then cooled to 45 ^{0}C, mixed thoroughly and poured into sterile petriplates.

Composition of rose bengal agar media:

Sr. No.	Ingredients	Quantity (g/litre)
1	Peptic digest of soyabean meal	5.0
2	Dextrose	10.0
3	Monopotassium phosphate	1.0
4	Magnesium sulphate	0.5
5	Rose bengal	0.05

Final pH (at 25^0C) 7.2 ± 0.2

The fungal strains used were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Fungal strains used were as follows:

1. *Aspergillus niger* (MTCC3017)
2. *Candida albicans* (MTCC1344)

(B) Preparation of test and standard solutions

Undiluted volatile oil in concentration of 5 µl. and 10 µl. was used as test oil. Fluconazole was used as the standard drug in the form of injection (Forcan,Cipla). It was diluted with water for injection to obtain required concentration of standard (25µg/0.1ml).

(C) Determination of zone of inhibition

The rose bengal agar, 20ml was poured into sterile Petriplates, under aseptic condition. The wells were prepared on agar surface by sterile cork borer of 6mm diameter. The test compounds of different concentrations (5 µl. and 10 µl.) were poured into the well with the help of micropipette and kept aside to allow the solution to diffuse totally in the medium. The plates were incubated at 25^0C for 48 hours in Biological Oxygen Demand (BOD) incubator (EIW Instruments Pvt. Ltd.). The zone of inhibition was measured in millimeters on antibiotic zone reader (Hally Instrument). Fluconazole (25 µg/ml.) was used as standard drug. Also, dimethyl sulfoxide was used as a control. All the experiments were performed in triplicates.

4.10 *in vitro* Antioxidant activity: [61,62]

DPPH radical scavenging activity

DPPH radical scavenging activity was measured by spectrophotometric method. 2 ml of methanolic solution of the extract of various concentrations (10-500 µg/ml) were mixed with 1 ml of methanolic solution of DPPH (1.5 mg/10ml). A mixture of 1 ml of methanol and 1 ml of methanolic solution of DPPH served as control. After mixing, all the solutions were incubated in dark for 20 min and then absorbance was measured at 517 nm.

In this method free radical scavenging potential of the extracts were tested against a methanol solution of 1,1 Diphenyl – β Picryl Hydrazyl (DPPH). Antioxidants react with DPPH and convert it to 1, 1 Diphenyl – β Picryl Hydrazine. The degree of discoloration indicates scavenging potentials of the antioxidant.

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical because of its unpaired electron delocalization over the whole molecule. The delocalization causes a deep violet color with substrate at 517 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable nonradical form of DPPH is obtained with simultaneous change of the violet color to pale yellow. The methanolic extract showed a concentration-dependent antiradical activity by inhibiting DPPH radical. They were able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorize 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability. It appears that they possesses hydrogen donating capabilities and acts as an antioxidant.

7. CONCLUSION

- During this study some variations related to morphology, microscopy, fluorescence analysis, phytochemical and physiochemical analysis were found among these species various parameters were establised successfully to compare all four species by taking these data.

- Morphologically these plants showed only a few differences while microscopically observed a lot of variations among these species. Epidermis in all species was found single layered pappilaneous with cuticle but in *O.citriodorum,* epidermis was found to possess vacuole like regions. Epidermis of *O. basilicum* has typical wavy cells. The type of stomata (diacytic) was found to be similar in all the four species. *O.bascilicum* has typical warty walled trichomes. The palisade cells of *O.sanctum* (Black) were longer than the other species, occupying more than half of the lamina.

- Evaluation of the physiochemical parameters was carried out & they were compared with that reported in the literature. Phytochemical screening was carried out & the major differences observed between *O.sanctum* (Green) & *O.sanctum* (Black) were the presence of alkaloids and terpenoids in *O.sanctum* (Black) while the same was not detected in *O.sanctum* (Green).

- For preliminary phytochemical analysis, volatile oil of all Ocimum species was subjected to TLC. Chromatographic separation for all volatile oils showed spots at various R_f values. HPTLC finger printing was established for volatile oils using Toluene: Ethyl acetate (93:7) as mobile phase. Subsequently, the chromatograms were scanned at 254 nm and 366 nm.

- *In vitro* Antibacterial, Antifungal and Antioxidant activity was performed and compared their activity to each other. Work on *O.citriodorum* was carried out first time. It is found to having potent antibacterial activity. Black variety of *O.sanctum* was showing highest antioxidant activity among these specien while *O.citriodorum* lowest.

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