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OCIMUM SANCTUM THERAPEUTIC POTENTIAL AGENT AGAINST ORAL PATHOGENS *E.FEACALIS* AND *S.MUTANS* ORGANISMS

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ABSTRACT

In this current study we have found that Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituents present in *O. sanctum* L. is largely responsible for the therapeutic potentials. The antibacterial activity combined with anti-inflammatory and analgesic activities of *O. sanctum*, could make it useful in inflammatory disorder resulting from microbial infection. Enterococcus faecalis *(E. faecalis)* is one of the most common species. Present in the mouth and vagina and Streptococcus mutans *(S. mutans)* is a facultatively anaerobic, gram-positive coccus (round bacterium) commonly found in the human oral cavity and is a significant contributor to tooth decay. It is reported in many literatures that *O. sanctum* L. extract has anti-cancer potential. due to the combinatorial effect of anti-proliferative, apoptotic and anti-migratory effect of the *O. sanctum* extracts.

***KEY WORDS*: *O. sanctum*, microbial activity, pathogen, bacteria.**

INTRODUCTION

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plants in Hindu culture is founds in "Rigveda", which is said to have been written between 4500-1600 B.C. and is supposed to be the oldest repository of human knowledge. It is Ayurveda, the foundation of medicinal science of Hindu culture, in its eight division deals with specific properties of drugs and various aspects of science of life and the art of healing. (Rastogi and Mehrotra, 2002) Medicinal plants are a source of great economic value all over the world. Nature has Best owed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country.

Tulsi is an important symbol of the Hindu religious tradition. Although the word 'Tulsi' gives the connotation of the incomparable one, its other name, Vishnu-priya means the one that pleases Lord Vishnu. Found in most of the Indian homes and worshipped, its legend has permeated Indian ethos down the ages. Known in English as Holy Basil and botanically called *Ocimum sanctum*.

Ocimum is a genus of about 35 species of aromatic annual and perennial herbs and shrubs in the family Lamiaceae, mostly native to the tropical and warm temperate regions of the Old World. Some medicinally important species includes (Steele, John.,2006).

Phytochemicals

Phytochemicals means "plant chemicals." Scientists have identified thousands of different phytochemicals, found in vegetables, fruits, beans, whole grains, nuts and seeds. Eating lots of plant foods rich in phytochemicals may help to prevent at least one in every five cases of cancer, as well as other serious ailments such as heart disease.

Phytochemicals (from the Greek word Phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (Hasler & Blumberg., 1999). They protect plants from disease and damage and contribute to the plant's colour, aroma and flavour. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals (Gibson et al. 1998 & Mathai.,2000). Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been catalogued (American Cancer Society) and are classified by protective function, physical characteristics and chemical characteristics (American Cancer Society) and About 150 phytochemicals have been studied in detail (American Cancer Society). In wide-ranging dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs and spices (Meagher E, Thomson C.,1999). Broccoli, cabbage, carrots, onions,

garlic, whole wheat bread, tomatoes, grapes, cherries, strawberries, raspberries, beans, legumes, and soy foods are common sources (Moorachian ME.,2000).

Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds (Costa MA.,1999). Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. Levels vary from plant to plant depending upon the variety, processing, cooking and growing conditions (King A, Young G.,2008). Phytochemicals are also available in supplementary forms, but evidence is lacking that they provide the same health benefits as dietary phytochemicals (American Cancer Society).

These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals. It is well-known that plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases (Narasinga Rao.,2003). Phytochemicals are not essential nutrients and are not required by the human body for sustaining life, but have important properties to prevent or to fight some common diseases. Many of these benefits suggest a possible role for phytochemicals in the prevention and treatment of disease, Because of this property; many researchers have been performed to reveal the beneficial health effects of phytochemicals.

MATERIAL AND METHOD

Collection of Plant Sample

Collection of plant material of *Ocimum sanctum* flower was successfully done from various area of Bhopal (M.P.).

Phytochemical Extraction & Screening

Processing of plant sample for Extraction

The flower of *Ocimum sanctum* after collection cleaned were allowed to dry in shade for 5-10 days and then grounded into fine powder in mixer grinder. 20 grams of dried powder was Subjected for Soxhlet extraction with different solvents for the extraction of phytochemicals.

Soxhlet Extraction

Preparation of Solvent system: Different solvents can be used depending on the kind of phytochemicals that are targeted for extraction. Solvents differ in polarity, just like phytochemicals. There are three polarity strengths of solvents and they are polar, medium-polar and non-polar. Polar solvents will extract polar chemicals and the same is true for non-polar solvents. Polar solvents include methanol, ethanol and water, medium-polar solvents examples are ethyl acetate, acetone and dichloromethane and non-polar solvents include toluene, chloroform and hexane. Thus, in a sample, different solvents can be mixed for extraction or they can be used in sequence in the same sample material.

For the extraction process in current work a solvents system of highest polarity is used i.e., pure Distilled Water which is non-volatile, the extraction of most of the polar phytochemicals from the samples for the study of their antimicrobial studies.

Test of Phytochemicals

A small portion of the dry extracts were subjected to the phytochemical test using Harborne's (1983) methods to test for alkaloids, tannins, terpenoids, saponins, flavonoids and glycosides.

1. **Test for alkaloids:** About 0.2 g extract warmed with 2% Sulfuric acid for two minutes, filtered and few drops of Dragendorff's reagent added orange red precipitate indicates the presence of alkaloids. And or filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.
2. **Test for glycosides:** The extracts hydrolysed with Hydrochloric acid solution and neutralized with Sodium hydroxide solutions. A few drops of Fehling solution A and B were added. Red precipitate indicates the presence of glycoside. Another test use was Benedict's test, in which the filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.
3. **Test for tannins:** small quantity of extracts mixed with water, heated, filtered and ferric chloride added. A dark-green solution indicates the presence of tannins.
4. **Test for saponins:** About 0.2g of the extracts shaken with 5ml of distilled water and then heated to boil frothing (appearance of creamy mix of small bubbles) shows the presence of saponins.
5. **Test for flavonoids:** Extract of about 0.2 g of the extracts shaken with 5ml of distilled water and then a few drops of 10% lead acetate solution is added. A yellow or dirty white precipitate shows the presence of flavonoids.

Isolation of Pathogenic Bacteria

Media preparation & Bacteria culture

A] Nutrient agar media: It's a general-purpose media which allows the growth of almost all bacteria, and it may be used to culture, subculture or storage of pure isolated bacterial colonies.

Composition of media; Beef extract 3 gm, Peptone 5gm, Sodium chloride 5 gm, and Agar 15 gm all the component dissolve in 1000 ml of distilled water.

B] Nutrient broth

Composition is; Beef extract 3 gm, Peptone 5gm, Sodium chloride 5 gm, all the component dissolve in 1000 ml of distilled water.

Sampling for Isolation of Bacteria

Reviving of Bacteria

All collected pure strains of testing bacteria *S.mutans*, *E.faecalis*, *E.coli* and *Pseudomonas aeruginosa*, were revived on nutrient agar media plates with quadrant streaking method. After the incubation all strains were taken for the partial identification.

Quadrant streaking method

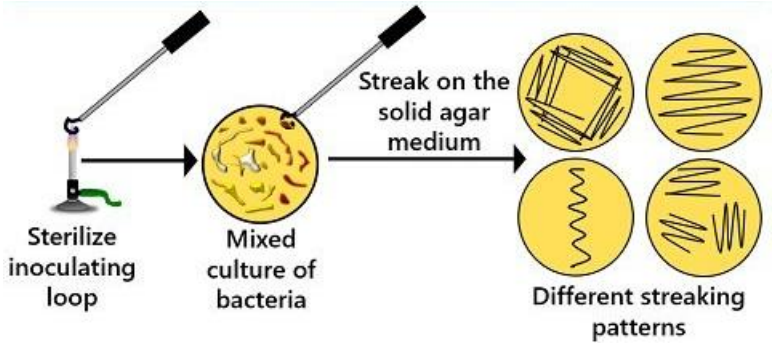

Fig 1: Streaking method for bacteria culture.

Identification of Bacteria

Colony morphology and microscopic study

Gram's staining procedure

Requirement: Crystal violet, Gram's iodine, Ethanol (95%) and Counter stain.

Procedure – Air dried or heat fixed bacterial smear was flooded with crystal violet staining. Reagent for 1 min. Then the smear is a gently washed in direct stream of tap water 2 second. After this side was flooded with mordant Iodine for 1 min now the slide is washed in a gentle and indirect stream of tap water for 2 second and the smear is immersed in 95% ethanol for 30 sec. with gently agitating. Now again smear is immersed with counter stain for 2 min. then smear is gently washed in to indirect stream of water until no color appeared in the wash water, and the smear is dried with absorbent paper and examined under microscope.

Biochemical test

1. IMViC TEST

The IMViC Test is a procedure used to differentiate Family Enterobacteriaceae**.** It consists of different biochemical test which stands for every letter in IMViC. "I" is for indole; "M" is for methyl red; "V" is for Voges-Proskauer, and "C" is for citrate.

1.1 Indole production test requirement

- Tryptone broth or peptone broth
- Kovec's reagent.

The Tryptone broth was inoculated with culture. Now 5 ml of 24 hours old culture was taken and 0.2 ml Of Kovec's reagent was added. A cherry red red color in the alcohol layer indicates the positive indole test.

1.2 Methyl red test

MR-VP broth: Composition; Peptone 5 gm, Dipotassium hydrogen phosphate 5 gm, Glucose 10% solution 50 ml all the component dissolve in 1000 ml of distilled water.

Methyl red indicator solution: Composition; Methyl red 0.1 gm, Ethanol 300 ml dissolves in 200 ml distilled water.

The tubes of MR-VP broth were inoculated with the test culture and incubated at 37^{o} C for 24 hour then after incubation 5-6 drops of methyl red solution was added. A bright red colour indicating a pH of 4.2 or less indicates positive test and yellow or orange.

1.3 Voges-proskauer test

MR-VP broth: Composition; Peptone 5 gm, Dipotassium hydrogen phosphate 5 gm, Glucose 10% solution 50 ml all component were dissolved in 1000 ml distilled water.

40% potassium hydroxide solution: The test culture was inoculated in the MR-VP broth and incubated at 37^{o}C for 48 hrs. After 48 hrs of incubation 1ml. of 40% potassium hydroxide and 3ml of a 5% solution of alpha-naphthol in a absolute ethanol was added. A positive reaction is indicated by the development of pink colour in 2.5 min becoming crimson in 30min. The tube can be shacked at intervals to ensure maximum aeration.

1.4 Simmons citrate test: Composition; Sodium chloride 5 gm, Magnesium sulphate 0.2 gm, Ammonium dihydrogen phosphate 1 gm, Potassium dihydrogen phosphate 1gm, Sodium citrate 5 gm, Agar 20 gm, Bromothymole blue 40 ml, pH 6.8 make up 1000 ml with distilled water.

Simmons citrate medium (a modification of koser's media with agar and an indicater) was dispense in test tube, then sterilized at 121^{o}C for 15 min and allowed to set as slope inoculate, saline suspension of the organism to be tested is inoculated on the agar slant. Then incubate for 24 hrs at 37^{o}C. A positive test shows a blue color on the streak of growth retention of original green color shows no growth on the line of streak indicates a negative test.

2. Catalase test - A nutrient agar slant was inoculated with the test culture or on any other medium lacking blood and incubated at 37°C for 24 hrs. following incubation 1 ml of 3 % H_2O_2 trickled was down the slant. Now examined immediately and after 5 min for the evolution of bubbles,which indicates the positive test.

Antimicrobial Studies

Broth cultures of the pure strains of *S.mutans* and *E faecalis*, were prepared by transferring a loop of culture into sterile nutrient broth and incubated at 37oC for 48hours. A loop full was taken from these broths and seeded onto sterile nutrient agar plates through sterile cotton swab to develop diffused heavy lawn culture.

The well diffusion method was used to determine the antibacterial activity and minimal inhibitory action of the extracts prepared from the *Ocimum sanctum* (Leaves)using standard procedure. In this method, first the test bacteria broth of bacteria are used to inoculate on the nutrient agar plates with the help of sterile cotton swabs to develop the lawn culture. Then to these plates 6 mm diameter well are punched in agar plates pre-inoculated with test microorganisms Undiluted overnight broth cultures should never be used as an inoculam. Routine direct application of suitably diluted extracts is poured into the well. The plates were incubated at 37oC for 24 hr. and then examined for clear zones of inhibition. Sterile water was used as control.

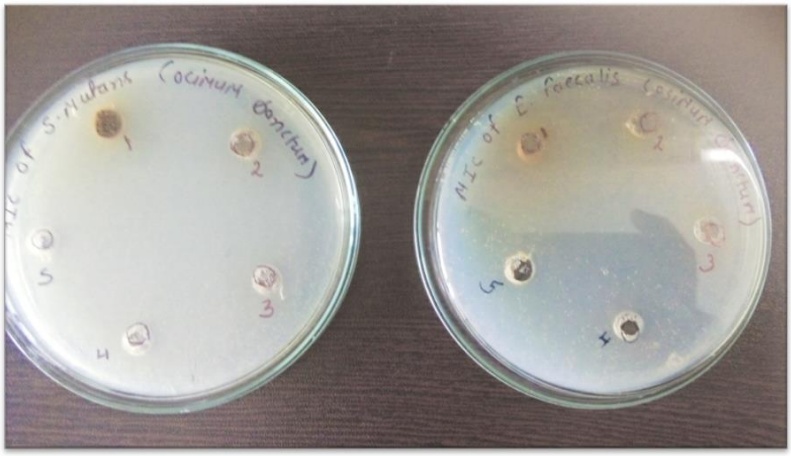

Fig 2: Anti-microbial activity.

RESULTS AND DISCUSSION

Sample Collection

Collection of plant material of *Ocimum sanctum* leaves was successfully done from resident area of Baghsevaniya Bhopal (M.P.)

Bacterial Isolation & Identification

For the studies of antimicrobial effect of phytochemical obtained from *Ocimum sanctum*, a medicinally important plant, the bacteria *S.mutans & E.faecalis*, were successfully obtained from the MPCST and on the basis of their colony morphology, microscopic characters, and partial identification we ensure the strain as pure. The results of the tests for bacterial identification are depicted in the table no.1. The isolates were taken for further experiments and antibiogram studies.

Table No. 1: Results of microbiological tests for selection of oral pathogens *S.mutans & E.faecalis.*

S. No.	Tests applied	Results	
		S.mutans	E.faecalis
1	Gram's staining	Positive coccus	Positive bacillus
2	Endospore Staining	Negative	Negative
3	Catalase	Positive	Negative
4	Indole production	Negative	Positive
5	Methyl red Reduction	Negative	Positive
6	VogusProskaur	Positive	Negative
7	Cirtrate Utilization	Negative	Negative

On the basis of partial identification, we used the bacterial strains for further studies.

Phytochemical Extraction & Screening

The phytochemical extraction of *Ocimum sanctum* barks was done successfully by drying t in shade, and grounding them into fine powdered material to increase its surface area to allow maximum solvent contact. This powder is them extracted with 40% acetone solvent systems to extract polar phytochemical in polar solvents. The whole process was done successfully in 250 ml capacity Soxhlet apparatus.

A small portion of the dry extracts were subjected to the phytochemical test using Harborne's (1983) methods to test for alkaloids, tannins, terpenoids, saponins, flavonoids and glycosides.

- **Test for alkaloids:** About 0.2 g extract warmed with 2% H_2SO_4 for two minutes, filtered and few drops of dragendoffs reagent added orange red precipitate indicates the presence of alkaloids.
- **Test for tannins:** small quantity of extracts mixed with water, heated, filtered and ferric chloride added. A dark green solution indicates the presence of tannins.
- **Test for terpenoids:** About 0.2 g extracts was mixed with 2ml chloroform ($CHCl_3$) and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish-brown coloration of the interface formed indicating the presence of terpenoids.
- **Test for saponins:** About 0.2g of the extracts shaken with 5ml of distilled water and then heated to boil frothing (appearance of creamy mix of small bubbles) shows the presence of saponins.
- **Tests for flavonoids:** Extract of about 0.2 g dissolved in diluted NaOH and HCl added. A yellow solution that turns colourless indicates the presence of flavonoids.
- **Test for glycosides:** The extracts hydrolysed with HCl solutions and neutralized with NaOH solutions. A few drops of Fehling solution A and B were added. Red precipitate indicates the presence of glycoside.

The results of phytochemical screening are deduced in table no.3.2

Table No. 2: Phytochemical screening of *Ocimum sanctum* acetonic extracts of leaves.

S. No.	Phytochemical Test	100% acetonic extract
1	Alkaloids	**Present**
2	Tannins	**Present**
3	Terpenoids	**Absent**
4	Saponins	**Absent**
5	Flavonoids	**Present**
6	Glycosides	**Absent**

Antibiogram studies

Since multidrug resistance of microorganisms is a major medical concern, screening of natural products in a search for new antimicrobial agents that would be active against these microorganisms is the need of the hour (Zgoda & Porter, 2001).

The present investigation in this project work, the antimicrobial activity of acetonoic extracts of *Ocimum sanctum* obtained from leaves was evaluated against *S.mutans* and *E.faecalis*. The fresh pure and diluted extracts obtained from plant barks were used on the test organism. Results of the experiment are being concluded in the Table 4.3, which clearly shows the anti-microbial activity of acetonic extract of *Ocimum sanctum* leaves extracts.

Table no. 3: Results of the antimicrobial activity of acetonic extracts obtained from *ocimum sanctum on oral pathogens S.mutans* & *E.faecalis.*

S. No.	Concentration	Zone of Inhibition in mm	
		S.mutans	E.faecalis
1.	100%	12	28
2.	50%	9	27
3.	25%	6	20
4.	12.5%	3	17
5.	6.25%	1	12

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay (Tona *et al.*, 1998).

Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants (Govindarajan *et al.*, 2006). Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings.

In present work the acetonic extract of *Ocimum sanctum* shows maximum antimicrobial activity against the test microbe *S.mutans* at each five concentration with zone of inhibition lying in the range 12 mm to 1 mm. but on the *E.faecalis* it shows higher inhibition at all concentration.

The results of present investigation and project work clearly indicate that the antibacterial activity occurs in

the extracts of plant material used. Thus, the study as certains the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

CONCLUSION

Our findings suggested that herbal preparation extracts have great potential as antimicrobial agent against microbes taken. Hence, it may be recommended that *O. sanctum* (Tulsi) plants could be used in the treatment against human diseases caused by the oral pathogens *E.feacalis* and *s.mutans* organisms.

In the present study we found that acetonic extraction of *O. sanctum* (Tulsi) has contains phytochemical alkaloid flavonoid and tannis mainly that has antimicrobial activity against oral pathogens like Enterococcus faecalis (*E. faecalis*) and Streptococcus mutans (*S. mutans*) is a facultatively anaerobic, gram-positive (round bacterium).

According to literatures it's also investigate that the *O. sanctum* (Tulsi) leaf and inflorescence extracts have the anti-cancer potential. This anti-cancer potential is due to the combinatorial effect of anti-proliferative, apoptotic and anti-migratory effect of the *O. sanctum* (Tulsi) extracts.

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