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***In vitro* Evaluation of Antibacterial Activity of Various Crude Leaf Extracts of Indian Sacred Plant, *Ocimum sanctum* L.**

Pankaj Goyal*¹ and Purshotam Kaushik¹

¹Department of Botany and Microbiology, Gurukul Kangri University, Harwar, Uttarakhand, India.

Research Article

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ABSTRACT

The medical world is on an immense requirement to discover novel antibiotics due to widespread emergence of resistance among microbial pathogens against currently available antibiotics. Traditional plants have been proved to be better source in the search for novel antimicrobial compounds. In such effort, we accessed the susceptibilities of some clinically significant bacterial species against various extracts made up from leaves of *Ocimum sanctum* L. (family: Lamiaceae). Antibacterial activity of crude extracts was found to be reliant on the nature of extract and the bacterial strains evaluated. Methanol extract was found to have comparatively higher activity than other organic and aqueous extracts. Gram-positive bacteria showed variable susceptibilities while Gram-negative *Salmonella typhi* has shown to be completely resistance to all the tested extracts. Minimum inhibitory concentration data showed hopeful results as some of the extracts exhibited significant inhibitions of bacteria even at low concentrations. This study indicated that leaves of *Ocimum sanctum* L. have significant antibacterial activity and it could be very useful in the discovery of novel antibacterial/antimicrobial agents.

Keywords: *Ocimum sanctum*; Indian sacred plant; resistance; antibiotics; natural products; antibacterial potential; susceptibility assay; minimum inhibitory concentration;

* Corresponding author: E-mail: pankaj.goyals@gmail.com;

ABBREVIATIONS

CFU: Colony Forming Unit; GC-MS: Gas Chromatography & Mass Spectrometry; IR: Infra-Red Spectroscopy; LC-MS: Liquid Chromatography & Mass Spectrometry; MDRTB: Multidrug-Resistant *Mycobacterium tuberculosis*; MHA: Mueller Hinton Agar; MIC: Minimum Inhibitory Concentration; mm: Millimeter; ml: Milliliter; MRSA: Methicillin-resistant *Staphylococcus aureus*; MTCC: Microbial Type Culture Collection; NCCLS: National Committee for Clinical Laboratory Standards; NCR: National Capital Region of India; NMR: Nuclear Magnetic Resonance; μ g: Microgram; μ l: Microliter; VRE: Vancomycin-resistant *Enterococcus faecalis*.

1. INTRODUCTION

In recent years, there has been a relentless increase in the occurrence of antibiotic resistance to many common bacterial pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* etc. Furthermore, methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Pneumococcus*, vancomycin-resistant *Enterococcus faecalis* (VRE), and multidrug-resistant *Mycobacterium tuberculosis* (MDRTB) are now commonplace pathogens that are proving difficult to treat effectively (Russell, 2002). Antibiotic resistance among bacterial pathogens is also increased in the hospital environment which is being considered in the context of its source, effects on individual patients and on hospital practice (Cohen, 1992; Gold and Moellering, 1996; Russell, 2002). This phenomenon of resistance among diverse bacterial pathogens against currently available antibiotics leads researchers to discover novel sources of antimicrobial drugs; thus, there has been a renewed interest in natural products from plants. These natural products would be able to provide a unique element of molecular diversity and biological functionality, which is indispensable for drug discovery (Perez et al., 1990; Nisbet and Moore, 1997).

Historically, plants have been placed at top among the sources of novel drugs with antimicrobial activity, as traditional medicines based on plants and plant extracts have made considerable contributions to human health and well-being. Plants provide a natural blueprint for the development of new drugs (Cragg et al., 1997; Iwu et al., 1999). It is estimated that plant materials have provided the models for more than 50% Western drugs today (Robbers et al., 1996). Plant based antimicrobials represent a vast untapped source for medicines and they provide enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials, and they offer more affordable treatment (Murray, 1995; Iwu et al., 1999).

India is a land of biodiversity in terms of plant species. Various plants have been mentioned in Ayurveda, an ancient Indian Sanskrit literature, for their therapeutic advantages (Kaushik, 1988). Many herbs used by Ayurvedic practitioners show promising results in the treatment of various ailments and these herbs could be appropriate for large randomized trials. Many potent drugs have been purified from medicinal plants having anti-rheumatic, anti-thrombotic, antimalarial, anticancer, antidiabetic and antimicrobial properties (Kaushik and Dhiman, 2000).

Tulasi (Holy Basil) is a traditional plant considered sacred by the Hindus. This religion links the plant with the Goddess figure as described in the *Puranas*. Hindus regard it as an earthly

manifestation of goddess Vrindavani, who is dear to Lord Vishnu. The name “*Tulasi*” in Sanskrit means “the incomparable one.” The *Shyama Tulasi* or Krishna Tulsi (*Ocimum sanctum* L. syn. *Ocimum tenuiflorum*) possesses great medicinal value as mentioned in *Charak Samhita*, an ancient Indian literature. It is a most common household plant in India and grows wild in tropics. Native to India, it is a short lived perennial herb or small shrub of Mint family Labiatae (Lamiaceae). It has small leaves with a strong smell and purple flowers. The foliage is green or purple, strongly scented. Oil extracted from leaves of this plant possesses significant insecticidal properties (Nanasombat and Lohasupthawee, 2005). *Ocimum sanctum* has been extensively studied for therapeutic potentials in various areas like immuno-stimulation, anticancer antioxidant, as adjuvant to radiotherapy, antiulcer, analgesic and antidiabetic (Hammer et al., 1999).

The present study aimed to investigate the susceptibility of several clinically significant bacterial strains against various crude extracts prepared from the leaves of *Ocimum sanctum* (Tulsi). The minimum inhibitory concentrations (MICs) were also determined for the crude extracts showing significant activity against the bacterial strains selected for the susceptibility assay.

2. MATERIAL AND METHODS

2.1 Collection of Suitable Plant Material

Ocimum sanctum (Figure 1) was collected in the month of May & June of 2007 from semi-arid, unshaded land of Ghaziabad (NCR), India. The plant was taken to the laboratory and was authenticated by Prof. P. Kaushik, Gurukul Kangri University. Leaves suitable for extraction were plucked and were washed under running tap water followed by sterilized distilled water. Leaves were air-dried, powdered and were subjected to the following extraction protocols.

2.2 Aqueous Extraction

Air-dried powder of *Tulsi* leaves (10 g) was boiled in 400 ml distilled water till one fourth of the extract, initially taken, was left behind after evaporation. The solution was then filtered using muslin cloth. Filtrate was centrifuged at 5000 rpm for 15 minutes. The supernatant was again filtered using Whatman filter no. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized bottles and stored at 4°C until further use.



Fig. 1. *Ocimum sanctum* plant

2.3 Organic Solvent Extraction

Air-dried powder (10 g) was thoroughly mixed with 100 ml organic solvent viz., ethanol, methanol, hexane or ethyl acetate. The mixture was placed at room temperature for 24 h on shaker with 150 rpm. Solution was then filtered through muslin cloth and then re-filtered through Whatman filter no. 1. The filtrate thus obtained was concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of various organic crude extracts were prepared by mixing well the appropriate amounts of

dried extracts and suitable solvent to give rise a final concentration of 100 mg/ml. Each solution was stored at 4°C after collecting in sterilized bottles until further use.

2.4 Test Bacteria for Susceptibility Assay

Tulsi extracts were evaluated for their antibacterial potential against both Gram-positive and Gram-negative bacteria. The bacterial strains were collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India. Bacterial species examined were *Escherichia coli* MTCC 739, *Salmonella typhi* MTCC 531, *Bacillus cereus* MTCC 430, *Bacillus subtilis* MTCC 736, *Streptococcus pyogenes* MTCC 442 and *Staphylococcus aureus* MTCC 740. These bacterial cultures were maintained in nutrient agar slants at 37°C. Each of the bacteria was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

2.5 Bacterial Susceptibility Assay

In vitro antibacterial activities of all aqueous and organic extracts from dried leaves of *Tulasi* plant were determined by standard agar well diffusion assay (Perez et al., 1990). Petri dishes (size 100 mm diameter) containing 18 ml of cool and molten Mueller Hinton Agar (MHA) (at 40°C) were seeded with 100 µl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 1.0×10^8 CFU/ml). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria inoculated. Wells of 6 mm diameter were cut into solidified agar media with the help of sterilized core-borer. Aliquot 100 µl of each extract was poured in the respective well and the plates were incubated at 37°C overnight. Organic solvents, in which extracts were prepared, were used as negative control while tetracycline antibiotic of one unit strength was used as positive control. The experiment was performed in triplicate under strict aseptic conditions. The antibacterial activity for each of the extract evaluated was expressed in terms of the average of the diameter of zone of inhibition (in mm) produced by the respective extract at the end of incubation period. Standard deviations were also calculated and represented in the respective table against each extract.

2.6 Determination of Minimum Inhibitory Concentration

Extracts producing an inhibition zone ≥ 15 mm in diameter were screened to determine minimum inhibitory concentrations (MICs) by standard two-fold microbroth dilution methodology given by NCCLS (1997). A stock solution of each active extract was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 8 µg/ml to 4096 µg/ml. A standardized inoculum for each bacterial strain was prepared so as to give inoculum size of approximately 5×10^5 CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain.

All the chemical ingredients used in present study were of analytical grade, and were purchased from Hi Media, India.

3. RESULTS AND DISCUSSION

Data of antibacterial activity of various crude extracts prepared from dry leave of *Ocimum sanctum* (Tulsi) are demonstrated in Table 1. Data indicated that the most active extract was

found to be of methanol which inhibited a total of four bacteria studied in the range of 11.86 mm to 18.50 mm size of inhibition zone. Ethanol, ethyl acetate, and aqueous extracts were found to be effective only against *Staphylococcus aureus* and *Escherichia coli* but, with comparatively lower activity than that of methanol extract. Hexane extract was found completely inactive against all the organisms tested.

Staphylococcus aureus (a Gram-positive bacterium) was observed as most susceptible bacterium as it was inhibited by almost all the extracts except hexane extract. *Streptococcus pyogenes* and *Salmonella typhi* was found to be resistant to all the extracts tested.

MICs of different active extracts from leave of tulsi had been demonstrated in Table 2. *Staphylococcus aureus* was inhibited at 1024 $\mu\text{g ml}^{-1}$ by methanol extract, while this bacterium was inhibited at 2048 $\mu\text{g ml}^{-1}$ concentration of ethanol and aqueous extracts. Inhibitions of *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* were not seen at even the maximum concentrations of ethyl acetate, ethanol and methanol extracts, respectively (i.e. MIC >4096 $\mu\text{g ml}^{-1}$).

Some earlier studies have found the inhibitory effect of methanol extracts of leaves of *Ocimum basilicum* in the range of 10 mg ml^{-1} against *Staphylococcus aureus* and 40 mg ml^{-1} against *Escherichia coli*. Our findings are in agreement with the results of these studies done with other species of *Ocimum* (Grosvenor et al., 1995; Navarro et al., 1996). In another study, ethanol extracts of *Ocimum sanctum* leaves have been found completely inactive against studied microorganisms (Nanasombat and Lohasupthawee, 2005).

It is in agreement with our investigation, since ethanol extract was found to have very weak activity against *Staphylococcus aureus* and *Escherichia coli* while other bacteria used in the current study were found to show complete resistance in this extract. The leaf extracts of *Ocimum gratissimum* prepared by using cold water, hot water, and steam distillation methods, were evaluated against *Salmonella typhi*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli*. Only the steam distillation extract inhibited the growth of the organisms with zone size ranging from 30 mm to 39 mm for different bacteria and MIC was evaluated just 0.1% for *Staphylococcus aureus* (Adebolu and Oladimeji, 2005).

The resistance of the organisms to the other extracts could be due to the evaporation of the oil during the boiling or due to insufficient release of the oil during cold extraction. The antimicrobial property present in the oil was probably due to the eugenol. Essential oil of *Ocimum gratissimum* was found significant inhibitory, while the oil from *Ocimum basilicum* and *Zingiber officinale* was found to have less activity (Nguefack et al., 2004).

Variations in the activity level might be attributed to the low affinity between less polar components of the plant extract and the polar substrate (agar). Opalchenova & Obreshkova (2003) observed the antibacterial effect of *Ocimum basilicum* (basil) essential oil against multidrug resistant clinical isolates of *Staphylococcus*, *Enterococcus* and *Pseudomonas* with MIC reported between 0.0030% and 0.0007% (v/v).

Basil oil has limited solubility in aqueous media, thus showing very limited activity in aqueous extract. Variable antibacterial activity in different extracts of *Ocimum* species against different bacterial strains were evaluated (Nakamura et al., 1999; Adiguzel, 2005; Mbata and Saikia, 2005).

Table 1. *In vitro* antibacterial activity of aqueous and organic extracts of *Ocimum sanctum* leaves

| Type of Extract | | Zone of Inhibition* (in mm diameter) | | | | | |
|------------------------|---------------|---------------------------------------|-------------------------|-------------------------------|------------------------------|--------------------------|------------------------|
| | | Gram-Negative Bacteria | | | Gram-Positive Bacteria | | |
| | | <i>Escherichia coli</i> | <i>Salmonella typhi</i> | <i>Streptococcus pyogenes</i> | <i>Staphylococcus aureus</i> | <i>Bacillus subtilis</i> | <i>Bacillus cereus</i> |
| Organic Extract | Ethanol | 12.50±0.50 | NI | NI | 13.83±0.58 | NI | NI |
| | Methanol | 14.67±0.58 | NI | NI | 18.50±0.87 | 12.70±0.75 | 11.86±0.81 |
| | Ethyl Acetate | 10.27±1.42 | NI | NI | 13.20±0.72 | NI | NI |
| | Hexane | NI | NI | NI | NI | NI | NI |
| Aqueous Extract | | 11.93±1.01 | NI | NI | 15.23±0.40 | NI | NI |
| | Tetracycline | 29.50±0.50 | 25.83±1.61 | 29.83±1.89 | 32.50±1.50 | 34.17±1.76 | 32.16±1.04 |
| Controls | Ethanol | NI | NI | NI | NI | NI | NI |
| | Methanol | NI | NI | NI | NI | NI | NI |
| | Ethyl Acetate | NI | NI | NI | NI | NI | NI |
| | Hexane | NI | NI | NI | NI | NI | NI |

*Mean of three values ± Standard Deviation. 'NI'-No Inhibition was observed.

Table 2. Minimum inhibitory concentration of active crude extracts of *Ocimum sanctum* leaves

| Type of Active Crude Extracts | Test Microorganisms | Concentration of Extracts* (in $\mu\text{g ml}^{-1}$) | | | | | | | | | | MIC (in $\mu\text{g ml}^{-1}$) |
|-------------------------------|------------------------------|---|------|------|-----|-----|-----|----|----|----|---|------------------------------------|
| | | 4096 | 2048 | 1024 | 512 | 256 | 128 | 64 | 32 | 16 | 8 | |
| Ethanol | <i>Staphylococcus aureus</i> | - | - | + | + | + | + | + | + | + | + | 2048 |
| Ethanol | <i>Escherichia coli</i> | + | + | + | + | + | + | + | + | + | + | >4096 |
| Methanol | <i>Bacillus subtilis</i> | + | + | + | + | + | + | + | + | + | + | >4096 |
| Methanol | <i>Staphylococcus aureus</i> | - | - | - | + | + | + | + | + | + | + | 1024 |
| Methanol | <i>Escherichia coli</i> | - | + | + | + | + | + | + | + | + | + | 4096 |
| Ethyl Acetate | <i>Staphylococcus aureus</i> | + | + | + | + | + | + | + | + | + | + | >4096 |
| Aqueous | <i>Staphylococcus aureus</i> | - | - | + | + | + | + | + | + | + | + | 2048 |
| Tetracycline | <i>Staphylococcus aureus</i> | - | - | - | - | - | - | - | - | - | - | <8 |

* (-) represents 'No Growth Observed'; (+) represents 'Growth Observed'

4. CONCLUSION

The current scenario of antibiotics is very threatening with significant emergence of resistance among bacterial pathogens against available antibiotics. The present investigation reveals that *Ocimum sanctum* and other plants would be the major source in finding metabolites with greater efficacy against resistant bacteria pathogens. The present study focused to the discovery of novel antibacterial/antimicrobials that can lead to the development of pharmaceuticals of plant origin. Since, the present study was focused only to examine the antibacterial potential of crude extracts; therefore further study can be made to isolate the pure compounds responsible for the activity from the extracts with the help of numerous advanced technologies such as GC-MS, LC-MS, IR and NMR.

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