ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2011; 2(4):1236-1242

ORIGINAL RESEARCH ARTICLE

Antibacterial Evaluation of Ethanolic Extracts of Four *Ocimum* Species against *E.coli* and *Salmonella abaetetuba*

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Received 08 May 2011; Revised 03 Aug 2011; Accepted 08 Aug 2011

ABSTRACT

In the present study, the antimicrobial activity whole plant ethanolic crude extracts of *Ocimum sanctum*, *O.basilicum*, *O.kilimandscharicum* and *O.gratissimum* at different concentrations (50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml, 250mg/ml and 300mg/ml) was assessed and tested against two Gram-negative bacteria i.e. *E.coli* and *Salmonell abaetetuba*. Ethanolic extracts of *O.basilicum* showed strong antibacterial activity against *E.coli* followed by other three *Ocimum* species in more or less in equal manners. Moreover *Salmonell abaetetuba* was more susceptible to *O.basilicum* in all concentrations tested. However other three species were effective only at higher doses at 200-300mg/ml. Phytochemical analysis and nutritional contents were demonstrated the common phytonutrients like phenols, tannins, glycosides, saponins, flavonols, alkaloids, fat, carbohydrate, protein, Ca, Fe, Na etc. These phytochemicals established a good support to the use of these plants in herbal medicine and as a base for development of new drugs.

Key words: *Ocimum sanctum*, *O.basilicum*, *O.kilimandscharicum*, *O.gratissimum*, Antibacterial activity.

INTRODUCTION

Natural products perform various functions, and many of them have interesting and useful biological activities [16]. There are more than 35,000 plant species being used in various human cultures around the world for medicinal purpose. The use of plant extracts and phytochemicals both with known antimicrobial properties can be of great significance in therapeutic treatments [34]. Many plants are used as medicines because of their antimicrobial nature, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances [12]. The demand on plant based therapeutics is increasing in both developing and developed countries due to growing recognition that they are natural products, easily biodegradable producing minimum environmental hazards, no side effects and easily available at affordable price. Researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs against cancer, as well as viral and microbial infections [16,17,38].

Tulsi is being used as medicinal herb for thousand years without any known adverse effects. Traditionally, juice of the leaves of *Tulsi* plant was used as demulcent, stimulant, expectorant. *Tulsi* is also used in the cure of upper respiratory tract infections, bronchitis, skin infections and earache [15]. An infusion of leaf had been used as anti-spasmodic in gastric disorders of children. A concoction of root of *Tulsi* is still being used as a diaphoretic in malarial fevers in remote areas. The seeds are mucilaginous and demulcent and are given in different ailments of genito-urinary system [3]. *Tulsi* is good for heart, stimulates digestion, and reduces breathing difficulties and cough [13]. It has also been used in the treatment of snake-bite and scorpion-sting as described in ancient texts by *Charaka* and *Sushruta*[19]. Thus, every part of the plant has useful application. Even today people use different parts of this plant for treatment of various ailments based on traditional knowledge (**Table 1**).

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The *Ocimum* (Basil) comprises some of the most popular herbs in the world. It belongs to the family Lamiaceae, sub-family Ocimoideae and includes 150 different species and varieties distributed in the tropical regions of Asia, Africa, Central and South Africa[6]. Because of its popularity basil is often referred to as the King of the herbs, being widely utilized due to its economic, nutritional, industrial and medicinal importance [18,35,36]. The genus *Ocimum* undergoes abundant cross-pollination resulting in large numbers of subspecies and varieties, which differ in essential oil composition and morphological characteristics.

In the present paper an attempt has been made to investigate the antibacterial activity of multi-solvent extracts of four *Tulsi* species against *Escherichia coli* and *Salmonell abaetetuba*. A preliminary phytochemical screening was conducted to identify the different classes of compounds present in the crude extracts of four *Tulsi* species.

Table-1: Traditional use of Four *Ocimum* species

Name of the Plants	Chemical Composition	Medicinal Uses	References
O.sanctum	It contains 0.5–1.5% essential oil of varying composition, about 5% tannins and p-sitosterol. Basil seeds contain planteose, mucilage, polysaccharides and fixed oil that consists of linoleic acid (50%), linolenic acid (22%), oleic acid (15%) as well as 8% unsaturated fatty acids. Basil leaves also contain 0.17% oleanolic acid and a small amount of ursolic acid. Also the leaves and flowers of *O.canum* Sims, contain oleanolic and ursolic acids and Possibly the seeds may also contain alkaloids and mucilage. A phenolic acid ester, triacontanol ferulate (ca. 0.01%) was identified from the stem bark of *O.sanctum.*	It has been suggested to possess antifertility, anticancer, antidiabetic, antifngal, antimicrobial, hepatoprotective, cardioprotective, analgesic and adaptogenic properties. *O.sanctum* and eugenol lowered cholesterol levels and enzyme activities induced by stress *Ocimum sanctum* extracts can be used to lure and trap fruit flies and so prevent damage to crops. A crude extract of *O.sanctum* showed pupicidal effect on newly emerged pupae of the vector *Aedes aegypti.*	List and Hörhammer 1977; Xaasan *et al.*, 1980; (Sukari *et al.*, 1995); Kumari *et al.*, 1994; Ramesh and Satakopan, 2010; Nahak and Sahu, 2011.
O.basilicum	Basil herb *(O.basilicum)* contains apart from essential oil (0.5–1.5%) also flavonoid glycosides (0.6–1.1%) and flavonoid aglycones. A flavone, xanthomicrol (5,4'-dihydroxy-6,7,8-trimethoxyflavone) was isolated from the leaves of a Nigerian *O.basilicum.* Three flavones, eriodictyol, eriodictyol-7- glucoside and vicenin-2 (apigenin di-C-glycoside), have been identified from the leaves of *O.basilicum* grown in Greece. The dried leaves and flower tops of sweet basil *(O.basilicum)* contain essential oil (ca. 0.08%), and protein (14%), carbohydrates (61%) and relatively high concentrations of vitamins A and C, rosmarinic acid and a flavone named xanthomicrol. The precursor of the cinnamates, *p*-coumaric acid, has been isolated and identified from the leaves of *O.basilicum.*	*Ocimum basilicum* has been used as an antiseptic, preservative, sedative, digestive regulator and diuretic. It also has been recommended for the treatment of headaches, coughs, infections of upper respiratory tract, kidney malfunction and eliminate toxin.	Viorica 1987; Fatope and Takeda 1988; (Skaltsa and Philianos 1990; Lang and Hörster 1977; Skaltsa and Philianos 1990; Leung and Foster, 1996; Nahak and Sahu, 2011.
O.kilimandscharicum	*O.kilimandscharicum* Guerke is rich in essential oil (2.5–7.6%) with 50–70% camphor as the main compound. The seeds contain fixed oil in which linoleic acid is the main fatty acid (50–70%).	The leaves treat congested chest, cough and cold, by sniffing crushed leaves or inhaling vapor of boiling leaves. Infusion is a cure for measles. It is also used to repel insects. Oil which has been partially decomphorized possesses insecticidal properties and may be used as a mosquito repellent' it is one-third as effective as pyrethrum extract/ it may be used also as a solvent for DDT.	Bown, Deni. 2001; Darrah, Helen, 1980; Tucker, Arthur and Thomas,. 2000; Wiersema, John and Blanca Leon, 1999; Nahak and Sahu, 2011.
O.gratissimum	Fresh leaves of *O.gratissimum* L.contain quite high amounts of essential oil (3.2– 4.1%).	The plant is considered digestive, tonic, stimulant, demulcent, diuretic, antimetic, antiseptic antidiarrhea and styptic. It is used in cough mixtures in combination with other expectorants. The plant is usedinthe treatment of epilepsy, high fever and diarrhea and also as a stomachic and laxative. The plant is used in treating coughs and fevers and as an anthelmintic. The leaves are eaten for stomach-ache. It is mosquito repellent and its cultivation is recommended as a means of controlling measure for this pest.	El-Said *et al.*, 1969; Onajobi, 1986; Oboh *et al*.,2009; Adebolu and Oladimeji, 2005; (Nahak and Sahu, 2011).

MATERIALS AND METHODS

Plant Materials

The leaves of four *Ocimum* species (*O.sanctum*, *O.basilicum*, *O.kilimandscharicum* and *O.gratissimum*) were collected from the local area of Baipriguda, Ambaguda, Boriguma, Sasahandi and Jeypore of Koraput district (India) in the month of September 2009.

Preparation of Crude Extracts

The surface of the leaves were washed in distilled water to remove the surface micro flora and are then shade dried, pulverized by a mechanical grinder and passed through a 40 µm mesh sieve to

get the fine powder and stored in an airtight container. The dried powder (50g) was extracted by soxhlet extraction method using ethanol as an organic solvent. Then the solution was concentrated in a rotary vacuum evaporator at reduced pressure below 40^0C and the extracts thus obtained were stored in airtight bottles at 4^0C till required for analysis.

Growth and Maintenance of Test Microorganism

Bacterial cultures of *Escherichia coli* (B)-ATCC3848 and *Salmonell abaetetuba*-ATCC35640 were obtained from the culture collection centre, Department of Microbiology, Orissa University of Agriculture and Technology, Odisha, India, were used for antimicrobial test organisms. The bacteria were maintained on nutrient broth (NB) at 37^0C.

Qualitative tests for phytochemical analysis

The phytochemicals in the plants were investigated by performing standard qualitative tests for the presence of alkaloids, flavanoids, saponins, tannins, terpenoids, steroids, lactones and carbohydrates were evaluated according to the methods described[9].

Antimicrobial Activity Assay

Disc Diffusion Test

The *in vitro* antimicrobial activity of the sample solution was done by disc diffusion method [31]. The crude methanol and fractionated extracts were dissolved in Dimethyl Sulfoxide (DMSO) with the exception of the water fraction and then antimicrobial effect of crude methanol and fractionated extracts were tested using two different concentrations. Petri dishes (measuring 90 mm each side) containing 20mL of Mueller Hinton agar (OXOID). At the same time, 6 mm diameter sterile Whattman antibiotic disc were placed on the surface of the inoculated agar plates, and then appropriate concentration of the extracts in DMSO and water were applied onto the discs, 50 and 300 mg/ml final concentrations were obtained for each discs. The plates were incubated at 37^0 C for 16-18 h. The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. Standard discs of the antibiotic Gentamycin (10µg), Ampicilin (10µg) and Tetracycline (30µg) served as the positive antibacterial controls. Negative controls were done using paper discs loaded with 20µL of DMSO and water. After that, the diameter of inhibition zone was measured in millimeters by Vernier Calipers. All tests were repeated three times to minimize test error. An inhibition zone of 14 mm or greater (including diameter of the disc) was considered as high antibacterial activity.

Results and Discussion

The identification of biologically active compounds is an essential requirement for quality control and dose determination of plant based drugs. A medicinal herb can be viewed as a synthetic laboratory as it produces and contains a number of chemical compounds. These compounds responsible for medical activity of the herb are secondary metabolites [8]. The results of phytochemical analysis of ethanolic extracts of four *Ocimum* plants showed positive results for alkaloids, anthraquinone glycosides, gums mucilage, proteins, carbohydrates, fat, tanins, phenol, triterpenoids, steroids, sterols, saponins, flavones, flavonoids and negative results for cardiac glycoside and thiol group (**Table 4**). The presence of alkaloids, saponins, tannins and flavonoids were detected in the plant. Some of these metabolites may be responsible for the antimicrobial activity exhibited [33].

The most potent effect related to ethanolic extracts of all four *Ocimum* species showed inhibition effect against *E.coli* (4-20mm) in different concentrations ranging from 50-300mg/ml. Antimicrobial effect against *Salmonella* is not uniform at different concentrations (50-300mg/ml). *O.basilicum* is more effective towards *Salmonella* in all concentrations where as *O.sanctum*, *O.kilimandscharicum* and *O.gratissimum* only inhibit at 250-300mg/ml. Results revealed that *E.coli* was the most susceptible bacterium which confirmed the results of disc diffusion method.

Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. *In vitro* studies in this work showed that the plant extracts inhibited bacterial growth but their effectiveness varied. The antimicrobial activity of many extracts have been previously reviewed and classified as strong, medium or weak [26].

Specifically previous work on *O.santum* reveals MIC of *Staphylococcus saprophyticus* was 10.19±0.08mg/ml and *S.aureus* was 18.68±0.95mg/ml [21]. Similarly it showed lower zone of inhibition to *Proteus vulgaris* and *Klebsiella pneumonia* [32]. Aqueous extract of *O.gratissimum* inhibited the growth of gram positive and gram negative bacterium (*E.coli*)[27]. *O.basillicum* extract was tested against 146 microbial organisms. Ethanolic extracts showed an inhibition range of 13-14mm against *E.coli* [2]. In study chloroform extracts, alcoholic extracts

and Oil from *O.sanctum* against *E.coli* was found effective which agrees to our experiment also [25].

The phytochemical analysis of some medicinal plant extracts revealed the presences of phytochemical which are known to be form biologically active. In this study phytochemical analysis of active extract demonstrated the presence of common phytonutrients like tannins, glycosides saponins, flavonoids and alkaloids. These are believed to be responsible for the observed antibacterial effects. Some studies have also attributed to their observed antimicrobial effect of plant extracts to the presence of these secondary plant metabolites. These pytochemical have been found to form irreversible complexes with proline-rich proteins [14]. Since flavonoids are known to be synthesized by plants in response to microbial infection [7]. The presence of tannins suggests the ability of this plant to play a major role as antidiarrhoea and antihaemorrhagic agent [29]. Presence of saponins revealed immense significance as antihypercholestrol, hypotensive and cardiac depressant properties [4]. The presence of cardiac glycosides has been used for over two centuries as stimulants in case of cardiac failure [38]. The differences in the antimicrobial effects of the plant extracts might be due to the phytochemical properties. It is quite possible that some of the plants that were ineffective against certain bacteria may have contained antibacterial constituents, just not in sufficient concentrations so as to be effective. This perhaps justifies the already locally established function of the plant in the treatment and management of hypertension. The presence of these phytochemical bases in *Ocimum* species accounts for their usefulness as medicinal plants.

In addition to secondary metabolites all the four *Ocimum* species contains little fat, higher carbohydrate content and moderate amounts protein, calcium, sodium and iron etc (**Table 3**). The nutritional properties of *Ocimum* species also play a role in antibacterial activity of *Ocimums*[27].

In the present study both these organisms are gram negative bacteria. Among these two micro-organisms *E.coli* was the most susceptible to ethanolic extracts of all four *Ocimum* species. All the four species of *Tulsi* included in the present study were found to be active against both the selected microbial strains. The antimicrobial activity profile of all species of plants against the tested strains indicated that *Escherichia coli* was the most susceptible bacterium and *Salmonell abaetetuba* was the most insensitive strain against all the doses (50-300 $mgmL^{-1}$) of all the the four species. While comparing between the two antimicrobial activities, a significant result was found in *E.coli* which has a great medical significance in causing gastroenteritis, urinary tract infection, pyogenic infections, septicemia etc. *S.abaetetuba* a facultative anaerobic gram negative bacteria which often infects cattle, poultry and domestic cats and have also been sources for infection to human causing food poisoning and diarrhea.

Our current study revealed that MIC of *E.coli* 50mg/ml in case of *O.basilicum*, 200mg/ml for *O.gratissium*. However in case of *S.abaetetuba* the MIC was 150mg/ml for *O.basilicum* and 250mg/ml for other three *Ocimum* species. The responses were very closing to Gentamycin (10µg), Ampicillin (10µg) and tetracycline (30µg) taken as standard antibiotics.

An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death [32]. These findings support the traditional of local users and it is a preliminary, scientific, validation for the use of these plants for antibacterial activity to promote proper conservation and sustainable use of such plant resources. Awareness of local community should be enhanced incorporating knowledge with scientific findings. In conclusion, the results of the present study support the folkloric usage of the *Ocimum*. This antibacterial study of the plant extracts demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use this plant in folk medicine suggests that they represents an economic and safe alternative to treat infectious diseases.

Table-2: Antimicrobial activity of Ethanolic extracts of Four *Tulsi* Species against *Escherichia coli* and *Salmonell abaetetuba*

Doses (mg/ml)	*O.sanctum*		*O.basilicum*		*O.kilimandscharicum*		*O.gratissimum*	
	(Diameter of inhibition zone in mm)							
	E.coli	*Salmonella*	*E.coli*	*Salmonella*	*E.coli*	*Salmonella*	*E.coli*	*Salmonella*
50	5	--	15	5	05	--	4	--

100	6	--	16	5	06	--	5	--
150	6	--	18	16	15	--	7	7
200	15	5	18	17	16	--	8	7
250	15	16	20	19	18	18	8	15
300	16	18	22	20	18	20	16	16
Gentamycin (10□g)=17mm								
Ampicilin (10□g)=15mm								
Tetracycline (30□g)=18mm								

Table-3: Nutritional Facts of Four Different Species of *Tulsi*

Nutrient Values (mg/100gm)	*O.sanctum*	*O.basilicum*	*O.kilimandscharicum*	*O.gratissimum*
Total Calories	4.32	3.96	2.87	2.43
Total Fat	0.04	0.03	0.03	0.02
Total Carbohydrate	57.52	43.63	36.45	30.15
Protein	12.6	10.2	9.14	8.5
Calcium	2923	2354	2015	1890
Iron	18	15	12	10
Sodium	154	120	112	97

Table-4 Qualitative Detection of Ethanolic Extracts in Various *Ocimum* Species

Phytochemicals	*O.sanctum*	*O.basilicum*	*O.kilimandascharicum*	*O.gratisimum*
Alkaloids	+	+	+	+
Cardiac glycoside	-	-	-	-
Anthraquinone glycosides	+	+	+	+
Gums mucilage	+	+	+	+
Proteins	+	+	+	+
Carbohydrate	+	+	+	+
Fat	+	+	+	+
Tanins	+	+	+	+
Phenolic compound	+	+	+	+
Triterpenoids	+	+	+	+
Steroids	+	+	+	+
Sterols	+	+	+	+
Saponins	+	+	+	+
Flavones	+	+	+	+
Flavonoids	+	+	+	+
Thiol group	-	-	-	-

+ = Present and - = Absent

IJPBA, July - Aug, 2011, Vol. 2, Issue, 4

ACKNOWLEDGEMENT

The authors are thankful to University Grants Commission, New Delhi for financial Assistance. We are also thankful to Principal of B.J.B. (A) College and H.O.D. of Microbiology Department, O.U.A.T., Bhubaneswar, Odisha, India for laboratory facilities for carrying out the experimental work.

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