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PHYTOCHEMICAL ANALYSIS AND EVALUATION OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF *VITEX NEGUNDO* AND *OPHIORRHIZA MUNGOS*

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ABSTRACT
Phytochemical analysis of the crude extracts revealed the presence of various primary and secondary metabolites. Nine bacteria including both gram positive and gram negative, (*B. subtilis, L. lactis, S. aureus, M. luteus, P. aeruginosa S. typhimurium, K. pneumoniae, P. vulgaris*, and *E. coli*) were tested for susceptibility to the extracts using disc diffusion method. The results were compared with antibiotic ampicillin which was used as the standard. Potent antimicrobial activity was shown by ethyl acetate extract and methanol extract of leaf and stem of *O. mungose* (sensitivity zone - 19 – 28mm for Ethyl acetate and 11 – 26mm respectively) and ethanol extract of leaf and stem of *V. negundo* (sensitivity zone - 9 – 16mm). The methanol extracts of leaf and stem of *O. mungos* and *V. negundo*, were analyzed using methods such as analysis of total phenolic content, reducing power and DPPH free radical scavenging assay for antioxidant activity determination. The highest free radical scavenging was shown by *V. negundo* stem (96.47% at 100 μg/mL), nearest to that of the ascorbic acid (97.44% at 100μg/mL) which was used as the standard. The activity shown by *O.mungos* stem was considerably lower with the least activity of 4.06% in the concentration 20 μg/mL.

INTRODUCTION

Mankind has been using plants as therapeutic agent for thousands of years and continues to rely on them for health care. The medicinal value of most of the plants lie in some chemical substances that they produce as secondary metabolites which has a physiological action on the human body. These ingredients have been proved to be useful in the treatment of chronic as well as infectious diseases. Plants and plant based medicines are the basis of many of the modern pharmaceuticals we use today for various ailments. For the present study, leaf as well as stem of two plants, *Ophiorrhiza mungos* and *Vitex negundo* was selected.

Ophiorrhiza mungos belongs to the family Rubiaceae which comprises 150 species, distributed throughout India, in shady places on planes and hills of low elevations. The roots of *Ophiorrhiza mungos* have been reported to produce Camptothecin (CPT) and 10-methoxycamptothecin (Tafur *et al*., 1976). Roots of the plant are bitter, acrid, thermogenic and sedative. They are useful in wounds, ulcers, snake poison, gastropathy, leprosy, helminthiasis and cancer. The aqueous extract of *O. mungos* root is reported to have anti-venom activity**.** *Vitex negundo* belongs to family Verbenaceae which comprises of 75 genera and nearly 2500 species, (Sastri, 1950; Nasir and Ali, 1974). The whole part of *Vitex negundo* is detected to have medicinal properties and is used for various ailments in indigenous system of medicine. Every part of this plant is valuable in medicine and various preparation of plant has been mentioned in indigenous system of medicine for various skin diseases, (Amann, 1975) and as nerve sedative, (Perry, 1980). Activities such as antibacterial, (Kustrak *et al*., 1987), anti-fungal, (Aswar *et al*., 2009) anti-inflammatory, anti-androgenic, (Bhargava, 1989) etc. have been reported from the plant. They are of high value as constituents of Ayurvedic preparations such as 'Vishagarphathaila', which is widely used to treat rheumatism in India, (Jayaweera, 1980). The chloroform extracts of detached seeds of *V.negundo* showed anti-inflammatory activity, mosquito repelling activity, (Asaka and Rana, 1973), and antitumor activity, (Horowitz and Gentili., 1966). It has hepato protective action against CCl_4 which induces liver damage, and has analgesic activity (Gupta *et al*., 1999).

The antimicrobial activities of plants have long been exploited by scientific community against the disease causing microbes. Drug resistance is a serious issue experienced as microbes are getting resistant to the currently used antibiotic agents. So the detection of new natural compounds with antimicrobial activity is considered to be important. Although hundreds of plant species have been said to have antimicrobial properties, the vast majority have not been adequately evaluated,

(Balandrin *et al*., 1985). So in this study the various extracts of stem and leaves of the two plants *O.mungos* and *V.negundo* were subjected to antibacterial assay against nine organisms. Plants produce various antioxidant compounds to counteract reactive oxygen species (ROS) in order to survive, (Lu and Foo, 1995; Velioglu *et al*., 1998 and Hudson, 1990). These ROS's are exacerbating factors in cellular injury and aging process, (Gupta *et al*., 1999). Literature revealed that plant parts extracted with methanol has cosiderable antioxidant property, (Krishnaraju *et al*., 2005). Hence, the methanolic extracts of both the plants were subjected to antioxidant assay using three standard methods such as determination of total phenolic content, reducing power antioxidant assay and free radical scavenging assay using DPPH.

MATERIALS AND METHODS

Plant material

The whole plant of *Ophiorrhiza mungos* were collected from Nedumangadu and Department of Botany, University of Kerala, Kariavattom campus. The stem and leaf of *Vitex negundo* were collected from Sasthamangalam. The materials were cleaned thoroughly with distilled water. The washed plant materials were air dried in shade.

Preparation of plant extracts

The dried plant parts were separately powdered in a grinder and sieved. The extraction of the plant material was performed using Soxhlet apparatus. The powdered plant materials were filled in the sample holder, and serially extracted using solvents such as hexane, chloroform, ethyl acetate, methanol and ethanol. The dried extracts were stored in clean air tight storage vials.

Phytochemical screening

The prepared crude extracts were qualitatively tested for the presence of chemical constituents. Phytochemical screening for the of various constituents such as, carbohydrates, proteins, phenolics, flavaniods, steroids, sterols, triterpines, tannins, terpenoids, glycosides, gums, resins, phlobatinins, saponins, and reducing compounds. Some of the tests were done using aqueous extracts alone as indicated in standard procedures, (Singleton and Rossi, 1965).

Table 1: Presence of phytochemicals in the crude extracts

Metabolites	*O. mungos* leaves	*O. mungos* stem	*V. negundo* leaves	*V. negundo* stem
Carbohydrates	+	+	+	+
Proteins	+	+	+	+
Phenolics	+	+	+	+
Flavanoids	-	+	+	+
Steroids	+	+	-	+
Sterols & Triterpine	+	-	+	+
Tannin	+	-	+	+
Terpenoid	+	+	+	+
Glycosides	+	+	+	+
Gums	-	+	-	-
Resisns	-	-	+	+
PhlobatanninS	-	-	+	+
Reducing compounds	+	+	+	+
Anthraquinones	-	-	-	-
Saponins	+	+	+	+

Microorganisms

The reference strain of nine bacteria (four gram positive and five gram negative) were procured from MTCC, *viz*. *Bacillus subtilis* (MTCC *121), *Lactococcus lactis* (MTCC 440), *Staphylococcus aureus* (MTCC 3160), *Micrococcus luteus* (MTCC *106), *Pseudomonas aeruginosa* (MTCC (424), *Salmonella typhimurium* (MTCC 98), *Klebsiella pneumonia* (MTCC 3384), *Proteus vulgaris* (MTCC 426) and *Escherichia coli* (MTCC 40).

Antibacterial activity

Antibacterial activity of the plant extracts (hexane, chloroform, ethyl acetate, methanol and ethanol extracts for *O. mungos* and hexane, ethyl acetate, methanol and ethanol extracts for *V. negundo*) at a concentration of 500μg/mL were checked by disc diffusion method according to standard method, (Bauer *et al*., 1966). The nine bacterial cultures were used to lawn nine nutrient agar plates using sterile swabs. Sterile discs impregnated with the extracts were placed on the agar surface (four on one plate). The control discs impregnated with DMSO were placed in the centre. The plates were then incubated at 37ºC for 24h. After incubation, the plates were examined for zone of inhibition. The inhibition zones were measured and recorded.

Antioxidant assay

The methanolic extracts of the study materials were analyzed for antioxidant activity as per standard protocols.

Determination of total phenolic contents

The amount of total phenolics in extracts was determined with Folin–Ciocalteu reagent, Tafur *et al*., 1976) with slight modification using tannic acid as a standard. The total phenolic content was determined as mg of Tannic Acid Equivalent (TAE) using an equation obtained from the standard tannic acid calibration graph. The total phenolic content was fond out using standard calculation method.

Concentration of Test = (OD of Test/OD of standard) x (Concentration of standard/Volume of Test) x100.

Reducing antioxidant power

The reducing antioxidant power of plant methanolic extracts was determined. The reducing power was compared with standard, Butylated Hydroxyl Anisole (BHA).

DPPH free radical scavenging activity

The free radical scavenging activity of methanolic extract of the plant parts was quantitatively assayed using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic anti-oxidants, (Cao *et al*., 1997). Percentage scavenging of the DPPH free radical was measured using the following equation

% DPPH radical scavenging = [1-(As/Ac)] $\times$100,

A_c = absorbance of control

A_s = absorbance of sample solution.

Table 2: Zone of inhibition of different extracts of the study materials against the nine organisms

Microorganism	Inhibition zone (mm)																		
	O. mungos										*V. negundo*								Ampicillin
	leaf					stem					leaf				stem				
	H	CL	EA	M	E	H	CL	EA	M	E	H	EA	M	E	H	EA	M	E	
B. subtilis	8.5	11	25	8		15	9	16	18	8	11	12	19	12	7	11	9	9	31
L. lactis	0		20	9	7	19	13	9	26	8			8	13	-	20	10	15	21
S. aureus	8	18	18	10	10	27	10	16	11	12	10	11	9	14	7	9	9	15	31
M. luteus		27	28	-	8	15	-	-	15	15	9	11	12	15	-	9	-	16	10
P. aeruginosa		8	19	-	-	-	-	-	15	-	-	8	-	-	-	-	-	16	7
S. typhimurium		7	-	14	-	15	16	10	13	8	-	-	13	9	-	9	-	9	7
K. pneumoniae	8.5	-	19	8	9	10	8	10	15	13	9	10	27	13	-	-	7	13	23
P. vulgaris		-	20	10	9	8	29	-	11	15	9	11	10	11	-	9	-	11	13
E. coli		-	22	-	-	14	17	-	15	-	10	14	11	15	-	13	9	11	30

H – Hexane, CL – Chloroform, EA – Ethyl Acetate, M – Methanol and E – Ethanol

Table 3: % Free Radical Activity methanol extract at various concentrations and Ascorbic acid

Study Material	% Free Radical Scavenging activityat different concentrations				
	20μg/mL	40 μg/mL	60 μg/mL	80 μg/mL	100 μg/mL
V. negundo leaf	13.68	23.83	36.45	37.95	44.13
V. negundo stem	65.75	79.88	86.32	93.03	96.47
O. mungos leaf	17.39	29.83	40.51	52.96	65.58
O. mungos stem	4.06	9.09	10.06	12.34	15.27
Ascorbic acid(Standard)	72.02	85.08	86.58	97.09	97.44

RESULTS AND DISCUSSION

Phytochemical analysis

The results of the phytochemical analysis of various fractions of the two plants are tabularized in to a table. (Table 1) Carbohydrates, proteins, phenolics, terpenoids, glycosides, reducing compounds and saponins were found in all the parts of both of the plants studied, whereas anthroquinones were found to be completely absent. The presence of flavanoids, phenolics, terpenoids, anthroquinones, carbohydrates, steroids etc were reported in *Vitex negundo,* (Sastri, 1950).

Antimicrobial assay

The antimicrobial potential of the different extracts of the study materials were tested and the results are summarized in the Table 2. A potent activity was show by ethyl acetate fraction of the leaf of *O. mungos* (inhibition zone - 25mm). The only organism resistant to this fraction was *S. typhimurium*. A consistent activity was shown by the methanolic extract of the leaf of *O. mungos* against all organisms studied. The ethanolic extract of leaf and stem of *V. negundo* also showed a good range of activity against most of the organisms studied suggesting the detailed analysis of the photochemical contents of the said extracts to find out the active compound responsible. The antimicrobial activities are attributed to the presence of steroids and iridoids, (Perry, 1980). Antimicrobial properties have been reported in the plant (Renuka *et al*., 2008, Srinivas *et al*., 2010).

Differences in the antimicrobial effects of plants are due to differences in the phytochemical properties and differences among species, (Renuka *et al*., 2008). The study indicated that the ethyl acetate and methanol extracts were superior to all other solvent extracts tested. The less activity shown by some extracts of the plant parts can be due to the absence of biochemical with antibiotic properties or less concentration of antibacterial constituents which is not enough to make it effective. Almost all the extracts showed activity against at least one of the nine organisms tested, which indicates that the test samples contain biologically active ingredients. The knowledge of exact mode of inhibition of specific compounds which are present in the plant extract, may contribute to the successful utilization of such natural compounds for the treatment of infectious disorders like bacterial and fungal diseases.

Antioxidant assay

Determination of total phenolic content

Concentrations of phenolics in the extracts were found out using equation obtained from standard tannic acid calibration graph as well as by using calculation. The results are expressed as tannic acid equivalents.

From graph,

Concentrations of phenolics in *V.negundo* leaf: 30mg tannic acid /mL

Concentrations of phenolics in *V.negundo* stem: 15mg tannic acid /mL

Concentrations of phenolics in *O.mungos* leaf: 46mg tannic acid/mL

Concentrations of phenolics in *O.mungos* stem:17mg tannic acid /mL

The highest value of phenolic content indicates that the plant has high antioxidant activity. Raghavendra *et al*., 2010 reported a high total phenolic content in *V. negundo*.

Reducing power antioxidant assay

The results of reducing power antioxidant assay showed that among the four test samples analyzed, *V.negundo* stem extract have the highest activity comparable to that of the standard (BHA). *V.negundo* stem also showed comparatively good reducing power. The reducing power of *O.mungos* was less. The lowest activity was showed by *O.mungos* stem (Fig.1).

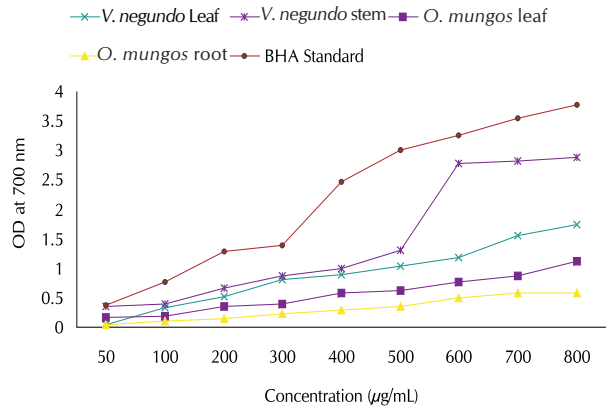

Figure 1: Results of reducing power antioxidant assay

Earlier works also showed the presence of good reducing power in V. negundo (Raghavendra *et al.*, 2010). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain or by donating a hydrogen atom Kumaran and Karunaka, 2007).

DPPH assay

The percentage of free radical scavenging assay was done on the methanolic extracts of the test materials. The results are summarized in Table 3.

The highest free radical scavenging was showed by *V. negundo* stem (100 µg/mL), nearest to that of the ascorbic acid which was used as the standard. The minimum activity was showed by *O.mungos* stem (20 ìg/mL). Methanol extract of *V. negundo* leaf also contained high amounts of bioactive compounds including total phenolic compounds. Similar observation was made by Kustrak *et al*. (1987). The plant is used as anti-inflammatory drugs by indigenous people. The radical quenching activity can be suggested as one of the method responsible for its anti-inflammatory activity, (Kustrak *et al*., 1987).

The antioxidant activity shown by *V. negundo* justifies the use of this plant as candidate for treatment of inflammatory disorders. The antibacterial activities shown by solvent fractions of both plants tested projects the potential of these plants for combating infections.

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