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In Vitro Antioxidant Activities of Ethanol and Petroleum Ether Extracts of *Anisomeles malabarica*

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A B S T R A C T

The present study was aimed to investigate the *in vitro* free radical scavenging activity of various root extracts (petroleum ether and ethanol) of *Anisomeles malabarica*. The free radical scavenging activity was found to be high in ethanol extract for DPPH, hydroxyl, and superoxide radical in a concentration dependent manner. Ethanol extract of root possessed antioxidant activity near the range of BHT which could be an essential rich source of natural antioxidant. Similarly in antibacterial screening, ethanol extract of root show notable antibacterial activity against tested microorganisms.

***Key words*:** Phytochemicals, Anisomeles, Antibacterial, GC-MS, Crude extracts

Natural products have long been implemented as alternative health care treatment and in discovery of modern drugs. Medicinal plants refer to the class of plants applied for therapy or to possess pharmacological actions for human and animal [1]. Drug discovery from traditional medicinal herbs have played a significant role in the therapeutic management of cancer and, undoubtedly, most novel clinical applications of medicinal plants and its secondary metabolites and derivatives of the medicinal plants over the last five decades have been effectively applied against combating cancer and other diseases [2].

Recently, much attention has been paid to the extracts obtained from plant species in order to analyze their biological activities. The discovery of novel secondary metabolites from medicinal plants is an important alternative to overcome the increasing levels of drug resistance by human pathogens. Due to the World's urgent need for new antibiotics and chemotherapeutic agents, growing interest is taken in to the research on the chemistry of medicinal plants [3].

Human body possesses numerous antioxidant defense systems and it comprises of enzymatic and non-enzymatic pathways which could help to maintain a steady equilibrium between prooxidants and antioxidant to ensure well-being [4]. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory [4-5]. The mechanism of action and efficiency of herbal extracts in most cases are yet to be scientifically validated. Therefore, significant work has been carried out by researchers to focus their attention towards traditional medicines for the development of outstanding drugs against different varieties of microbial infections. Phytochemicals are more promising than synthetic chemicals due to their generation from living organisms, potential biological actions and innate stereo-chemistry allowing their binding to protein pockets [6].

Traditionally, herbal medicinal preparations are derived from plants, whether in the simple form as plant parts or as crude extracts or mixtures, among others. Today, considerable numbers of drugs are developed from plants which are active against a number of diseases [7]. After many years, isolation of active compounds such as morphine, quinine and alkaloids ushered in the dawn of a new era in the use of medicinal plants and marked the beginning of present in the use of plants to cure diseases [8-9].

Description of Anisomeles malabarica

The whole plant, especially the leaves and the roots are used as astringent, carminative, febrifuge and tonic. Treatment with medicinal plants is considered very safe as there is no or minimal side effects. These remedies are in sync with nature, which is the biggest advantage. The golden fact is that, use of herbal treatments is independent of any age groups and the sexes [10-11]. Since, *A. malabarica* showed significant antioxidant activity, it can be developed into a safe herbal formulation useful in stress and stress related disorders.

MATERIALS AND METHODS

Preparation of plant extracts

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The petroleum ether and ethanol extract was prepared using Soxhlet apparatus (100 g of powder in 500 ml of solvents). The root powder was extracted until the solvent becomes colorless in the thimble after which was filtered through Whatmann No.1 filter paper. It was concentrated using rotary evaporator (Heidolph, Germany) under reduced pressure and the residues were stored in amber colored glass vials at 4°C for further use.

Qualitative screening of phytochemicals

Screening for the presence of active phytochemicals in leaf extracts of *A. malabarica* was carried out using the standard method [12].

In vitro antioxidant activity

Free radical scavenging assay using DPPH

The free radical scavenging activity of each sample was determined using UV/vis spectrophotometer according to the method described by [13]. Briefly, 0.1 mM solution of DPPH in methanol was prepared and the initial absorbance was measured at 517 nm and did not change throughout the period of assay. An aliquot (20-100 µL) of an extract was added to 3 mL of methanolic DPPH solution. Ethanol alone served as blank and DPPH in methanol without plant extracts served as positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 517 nm. Radical scavenging activity was calculated as follows:

$$FRSA = [(A_c - A_s) / A_c] \times 100$$

Where, Ac - Absorbance of control and As - Absorbance of tested sample after 30 min.

Superoxide anion radical scavenging assay

Superoxide radical scavenging of different extract was performed with slight modifications [14]. Nitro blue tetrazolium (NBT) solution of 1 mL (156 µM), 1 mL of NADH solution (468 µM) and various concentrations of test samples (20–100 µg/mL) were mixed and the reaction started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM) prepared in phosphate buffer (100 mM, pH 7.4). The reaction mixture incubated at 25°C for 5 min and the absorbance was measure at 560 nm.

$$\text{Radical scavenging activity (\%)} = [(A_O - A_1) / A_O] \times 100$$

Where, A_O is absorbance of the control, A_1 is the absorbance of test samples

Hydroxyl radical scavenging assay

The scavenging ability of hydroxyl radicals (OH•) was measured [15]. Various concentrations of plant extract (20 – 100 µg/mL) was added to 1 mL of ion- EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%) and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80 – 90°C for15 min in water bath. After incubation, the reaction was terminated by addition of 1 mL ice-cold TCA (17.5% w/v). In which 3 mL of Nash reagent (7.5 g of ammonium acetate, 0.3 mL of glacial acetic acid and 0.2 mL of acetyl acetone was mixed and made up to 100 mL with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm using UV-Vis spectrophotometer. The reaction mixture without sample was used as a negative control whereas; BHT was used as a positive control. Percentage of scavenging ability (HRSA) was calculated using formula:

Hydroxyl radical scavenging activity (%) = 1 – A Sample – A Control × 100

In vitro antibacterial activity

Antibacterial activity of bioactive compoundwas determined using agar well-diffusion method. The bacteria used in the study were obtained from IMTECH, Chandigarh. The bacterial culture i.e., *Bacillus subtilis ATCC 23857, Escherichia coli, ATCC 23857 Micrococcus luteus ATCC 4698, Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853* were maintained in Mueller Hinton Agar (MHA) slants and used prior to assay.

Preparation of inoculums

The overnight broth culture of respective bacterial cultures were adjusted to turbidity equivalent to 0.5 McFarland standards (0.2 mL culture of organisms were dispensed into 20 mL sterile nutrient broth and incubated for 24 hours and standardized at 10^5-10^7 CFU/ml adjusting the optical density to 0.1 at 600 nm).

Procedure

Antibacterial activity of petroleum ether and ethanol extracts was determined using agar well-diffusion method. 0.1 mL of active growth culture was poured over feeder layer and spread evenly using sterile spreader. The 6 mm diameter well was made using a sterile cork borer. Each well received different concentrations (50, 100 and 150 µg/mL) of extracts. Appropriate control was maintained and the plates were incubated at 37°C for 48 hours. After incubation, the inhibition zone was measured [16].

RESULTS AND DISCUSSION

Phytochemical analysis

Quantitative analysis of root extract of *A. malabarica* exhibited higher flavonoid contents in ethanolic extract (18.04), phenols (15.62), saponins (17.21), tannins (12.03), alkaloids (3.40), glycosides (5.16) and Quinones (2.22) mg/g (Table. 1b). Quantitative phytochemical analysis revealed that, the presence of active principles such as flavonoid, phenols, saponins, alkaloid and carbohydrates in high amounts and steroids, cyanins and terpenoids in trace amount. Flavonoids as one of the most diverse and widespread groups of natural compounds are probably the most important natural phenolics which is considered to be very important plant constituents responsible for free radical scavenging ability owing to their hydroxyl groups [17].

Table 1 Quantitative analysis of phytochemicals from *A. malabarica*

Secondary metabolites	Ethanol (mg/g)	Petroleum ether (mg/g)
Alkaloids	3.40	1.53
Carbohydrates	2.16	1.05
Cyanin	1.650	4.34
Flavonoids	18.04	7.08
Glycosides	5.16	3.20
Phenols	15.62	4.78
Quinones	2.22	4.22
Saponins	17.21	3.51
Steroids	2.03	8.30
Tannins	12.03	4.49
Terpenoids	1.23	1.45
Triterpenoids	1.09	0.93

In vitro antioxidant assays

DPPH Free radical scavenging of A. malabarica

The petroleum ether and ethanol root extracts of *A. malabarica* were evaluated by their ability to scavenge DPPH radicals and was compared with standard. Highest radical scavenging was observed in ethanol extract (89.30±0.34, 73.40±0.45, 59.03±0.56, 40.10±0.89 and 33.03±0.34 at 20 - 100 µg/mL as well as in petroleum ether it was 68.14±0.32, 59.40±0.13, 48.90±0.51, 33.03±0.34 and 19.08±0.27at 20 - 100 µg/mL and petroleum ether extract revealed less scavenging activity, respectively when compared with standard Butylated hydroxyl Toluene 84.96±0.76 activity (Table 2).

Table 2 DPPH assay of *A. malabarica*

Concentration (µg/ml)	Petroleum ether	Ethanol	BHT
20	19.08±0.27	33.03±0.34	33.21 ± 1.2
40	33.03±0.34	40.10±0.51	49.90 ± 1.5
60	48.90±0.51	59.03±0.56	63.42 ± 0.46
80	59.40±0.13	73.40±0.45	79.89 ± 0.53
100	68.14±0.32	89.30±0.34	84.96 ± 0.76

The results obtained in this study suggest that all the extracts from *A. malabarica* showed radical scavenging activity by their electron transfer or hydrogen donating ability. There was a linear correlation between antioxidant activity and total phenolic content Polyphenol contents and tocopherols scavenge the DPPH radicals by their hydrogen donating ability [18-19].

Superoxide radical scavenging of A. malabarica

Two different solvent extract such as petroleum ether and ethanol was evaluated to identified the ability to scavenge hydroxyl radicals. Maximum radical scavenging was observed in ethanol extract (85.24±0.12, 72.01±0.52, 57.38±0.48, 39.16 ±0.79 and 23.41±0.14 at 20 - 100 µg/mL as well as in petroleum ether it was 59.20±0.21, 48.05±0.34, 38.09±0.56, 24.03±0.07 and 13.08±0.50 at 20 - 100 µg/mL and petroleum ether extract revealed less scavenging activity, respectively when compared with standard BHT 88.02±0.09 activity (Table 3).

Table 3 Superoxide scavenging of *A. malabarica*

Concentration (µg/ml)	Petroleum ether	Ethanol	BHT
20	14.08±0.27	23.41±0.14	38.21 ± 0.5
40	27.03±0.34	39.16±0.79	49.90 ± 0.68
60	38.90±0.51	57.38±0.48	66.12 ± 0.46
80	50.40±0.13	72.01±0.52	73.28 ± 0.53
100	65.14±0.32	85.24±0.12	88.02 ± 0.09

Ethanolic root extract of *A. malabarica* have greater scavenging activity than petroleum ether extract which might be due to the presence of scavenging compounds in extract. Generally, plants include considerable extents of phytochemical antioxidants such as flavonoids, phenolics, carotenoids, and tannins, which can be utilized to scavenge the extra free radicals existing in the body [20]. It has been recognized that the antioxidant effect of the flavonoids and their effectiveness on human health and nutrition are considerable. Earlier reports are revealed that, flavonoids are effective antioxidants mainly because they scavenge superoxide anions [21-23].

Hydroxyl scavenging of A. malabarica

The percentage of hydroxyl scavenging of *A. malabarica* and BHT were (Table 3). Increased scavenging activity (68.30%) in ethanol extract and (58.14%) in petroleum ether extract was obtained at low concentration (100 µg/mL).

Table 4 Hydroxyl scavenging of *A. malabarica*

Concentration (µg/ml)	Petroleum ether	Ethanol	BHT
20	11.28±0.35	16.03±0.20	31.81 ± 1.10
40	21.43±1.09	31.10±0.56	49.35 ± 0.92
60	39.06±0.68	44.03±0.47	63.92 ± 0.46
80	47.10±0.41	58.40±0.16	79.07 ± 0.53
100	58.14±0.23	69.30±0.71	89.31 ± 0.76

The antioxidative effect of *A. malabarica* root extract is mainly due tannins and phenols [24]. Our results are concomitant with previous findings where high content of phenolics in alcoholic extract of *Moringa oleifera* leaves compared to aqueous extract was reported [25]. In recent years much attention has been devoted to natural antioxidants and their health benefits. The high reactivity of hydroxyl radicals lead to tremendous damage to the cell and its components and subsequently to the organisms as a whole [26]. Therefore, it is very important to remove hydroxyl radicals which cause detrimental effects. Present result suggests that the ethanol extract exhibited more antioxidant agents than the petroleum ether extract. It was concluded that *A. malabarica* root contains some antioxidant agents that could be major factor for therapeutic potential against Reactive oxygen species related diseases.

Antibacterial activity of A. malabarica

The antibacterial activity of root extract of *A. malabarica* (ethanol and petroleum ether extract) is summarized in Table 4. Dose-dependent zone of inhibition (7 – 15 mm) is obtained against tested pathogens. The ethanol extract is more potent against both gram positive and negative pathogens at a lower concentration (100 µg/mL).

Few studies reported the antimicrobial property of *A. vulgaris* against *S. aureus* and *E. coli as well as P. aeruginosa* similarly to our finding [27-28]. The antibacterial activity of ethanol extract is associated with the presence of secondary metabolites which showed that the root of *A. malabarica* constitutes phytochemicals that possess antibacterial and antioxidant properties and could serve as a potential source in search of plant-based antibiotics and natural antioxidants.

Table 5 Antibacterial activity of *A. malabarica* against human pathogens

Extract	Zone of Inhibition (mm)														
	B. subtilis			*E. coli*			*M. luteus*			*S. aureus*			*P. aeruginosa*		
	µg/mL														
	50	75	100	50	75	100	50	75	100	50	75	100	50	75	100
P. Ether	-	7	8	-	7	10	-	7	8	-	-	7	-	7	9
Ethanol	-	7	11	-	8	15	-	8	12	7	8	11	-	8	13

CONCLUSION

Anisomeles malabarica is an important traditional medicinal plant, which possess rich source of phytocompounds. The results of the present study revealed that, ethanol extract illustrated the robust antibacterial activities against *E. coli, S. aureus and P. aeruginosa* and it could be used as antimicrobial agents to prevent many diseases in the near future. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Further, extensive research is required to identify and explore bioactive compounds from this plant.

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Conflict of interest

The authors declare no conflict of interest.

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