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Research Article

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An *in-vitro* anti-inflammatory and anti-oxidant activity of *Anisomeles malabarica* R.Br. Ex Sims

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ABSTRACT

In present study different solvent plant extracts and essential oil of Anisomeles malabarica R.Br. Ex Sims leaves and flowers were evaluated for In-vitro anti-inflammatory and anti-oxidant activity using different assay method. The In-vitro anti-inflammatory activities were analyzed by; Heat Induced Hemolytic and Protein Denaturation method using Diclofenac sodium as a standard. Anti-oxidant potencies were ascertained by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and Iron chelating methods. Ascorbic acid was used as a standard. The percentage inhibition and stabilization values for anti-inflammatory and scavenging and chelation values for anti-oxidant were obtained and tabulated. The result clearly indicates that the methanolic extracts of both flower and leave of the study species shows effective anti-inflammatory and anti-oxidant properties. This scavenging free radical capacity can be used as a good anti-oxidizing agent in various fields of medicine, food industry etc.

Keywords: In-vitro, Anti-oxidant, Anti-inflammatory, *Anisomeles malabarica*, Diclofenac sodium and Ascorbic acid.

INTRODUCTION

Since very old times, herbal medications have been used for relief of symptoms of disease [1]. Herbal medicines and products now find an ample use in today's world in order to meet health care needs and because of its less side effect nature. Hence, the majority of the populations continue to hold traditional medicines in high esteem. The acknowledgment to this, the World Health Organization has called the attention of many countries to the ever increasing interest of the public in the use of herbal medicines and encourages countries to identify and exploit those aspects of traditional medicine that provide safe and effective remedies [2].

Inflammation is a disorder involving localized increase in the number of leukocytes and a variety of complex mediator molecules. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation [3]. Previously, it was reported that reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion, and peroxynitrite radicals will participate in the process of inflammation [4]. These ROS will produce in excess will injure cellular bio molecules such as nucleic acids, proteins, carbohydrates and lipids, causing cellular and tissue damage and leads to different inflammatory conditions particularly, skin inflammations, broncho-inflammations and arthritis which augments the state of inflammation [5]. Therefore, the compounds showing scavenging activities towards these ROS may expect to have therapeutic potentials towards inflammatory diseases.

Antioxidants are chemical compounds that can scavenge free radicals that are formed in the body due to normal physiological process. These free radicals then initiate a chain reaction which leads to the formation of various other free radicals leading to oxidative stress which in turn results in the productivity of reactive oxygen species and reactive nitrogen species causing lipid peroxidation (LPO) and cellular damage [6]. The violation in the balance between oxidants and reductants in benefit for the oxidative processes is called as "oxidative stress." Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress. Large number of medicinal plants has been investigated for their anti-inflammatory and antioxidant properties as plants are good source of natural products such as polyphenols, alkaloids, flavonoids and other secondary metabolites. Flavonoids, a group of polyphenolic compounds isolated from plants are known to shows properties, such as free radical scavenging activity, inhibition of hydrolytic and oxidative enzyme and anti-inflammatory action.

Anisomeles malabarica R.Br. Ex Sims is an aromatic, densely pubescent, perennial herb, 1.2–2.0 m in height belonging to the family Lamiaceae. It is commonly found in Western Ghats from Maharashtra to Karnataka, Andhra Pradesh, Kerala and Tamil Nadu [7, 8]. The plant is reported to possess anti-periodic, diaphoretic, emmenagogue properties [9]. Ethno botanically, the leaves of the plant are used against convulsions, dyspepsia in intermittent fevers, colic, boils and tetanus [10]. The herb is also reported to be useful in inflammation, cough, cold, stomachache, itches and uterine affections [11]. *Anisomeles malabarica* R.Br. Ex Sims is also known to possess antifertility, antispasmodic, anticancer, diuretic, antimicrobial and anticonvulsant activities [12]. In-vitro anti-oxidant activity of methanolic extracts of *Anisomeles malabarica* R.Br. Ex Sims leaves were performed [13]. However to the best of our knowledge, the whole plant with respect to anti-inflammatory activity, different plant extracts and essential oils were not scientifically done for its In-vitro activity. Hence in this paper, the In-vitro anti-inflammatory effects of leaves, flowers extracts and essential oils of *Anisomeles malabarica* R.Br. Ex Sims were investigated. Furthermore, the study also evaluated the antioxidant scavenging activities of the different extracts and essential oil of the selected plant.

EXPERIMENTAL SECTION

Plant material collection:
The whole plant of *Anisomeles malabarica* R.Br. Ex Sims was collected from Dindigul district region of Tamil Nadu, India. The plant was botanically authenticated at Botanical Survey of India (BSI). A voucher specimen (MVS-1) of the plant has been deposited at the herbarium of the BSI, Pune.

Preparation of extract:
The leaves and flowers of *Anisomeles malabarica* R.Br. Ex Sims were washed, air and shade dried at room temperature for two weeks and made into fine powder using electric grinder and sieved using a 100 mesh sieve and used for further studies. Ten grams of the plant powder was packed in Whatmann filter paper. A successive solvent extraction was performed with solvents; water, methanol, chloroform, ethyl acetate and petroleum ether using Soxhlet techniques. The extracts were evaporated by rotary evaporator and the residue was used for further studies.The fresh plant material of the plant was subjected to essential oils were extraction by hydro- distillation method using Clevenger apparatus. The essential oil also treated to check anti-oxidant and anti-inflammatory activies.

Chemicals and Reagents:
The chemicals used were 1, 1-dipheny l-2-picrylhydrazyl (DPPH), Bovine Serum Albumin (BSA) was procured from Sigma-Aldrich (Germany). Ascorbic acid, Gallic acid was purchased from SD-Fine Chemical limited. Diclofenac sodium was obtained from Cipla pharmaceuticals (India). All other chemicals and solvents were procured from Merck India and are of AR grade and used as obtained without any purification.

Phytochemical Screening:
Phytochemical analysis revealed that methanolic extracts of the plant shows most of test positive. The detailed analyses of tests were tabulated (Table 1 and 2).

Determination of Anti-oxidant activity:
The anti-oxidant activities were evaluated by following methods.

DPPH radical scavenging assay [14]
DPPH (2, 2-diphenyl picryl hydrazyl) is a commercially available stable free radical, which is purple in colour. The anti-oxidant molecules present in the plant extracts, when incubated at room temperature, react with DPPH and convert it into biphenyl hydrazine, which is yellow in colour and shows maximum absorbance at 515nm. The test solution contains 0.5ml of 0.5mM solution of DPPH in methanol and 1.0mL of plant extracts. It was then kept at room temperature and after 15 minutes the absorbance of the test solution was measured. Similarly, absorbance of the control (DPPH and methanol) and standard ascorbic acid was measured. The DPPH radical scavenging activity of the plant extract was calculated as the percentage inhibition.

Iron chelating activity [15]
The variable oxidation states of transition metals and their ability to form a complex with organic compound find an application for determining redox property of plant extract. Iron (III) solution do not form complex with 1, 10-phenantroline solution only Iron (II) form a stable complex with the ligand 1, 10-phenantroline. When Iron (III) solution with some antioxidant will get reduce to Iron (II) and form complex with ligand which can be determined by spectrophotometrically at 515nm. In Iron chelating method; the test solution contains 1.0mL of 0.05M Ferric (III) ammonium sulphate, 0.5mL of 0.05% 1, 10-phenantroline solution and 1.0mL of plant extract. . It was then kept at room temperature and after 20 minutes the absorbance of the test solution was measured. Similarly, absorbance of the control (1.0mL of 0.05M Ferric solution+0.5mL of 0.05% 1, 10-phenantroline solution) and standard ascorbic acid was measured. The percentage metal chelating activity of the plant extract and standard were calculated.

Determination of Anti-inflammatory activity:
The anti-inflammatory activity was determined by the following method.
The membrane stabilization test was carried out for the selected plant leaves different extract.

Preparation of red blood cell (RBC) suspension
Fresh whole human blood (20.0mL) was collected and 2.0mL of anti-coagulating agent 1% EDTA solution was added and were centrifuged at 3000 rpm for 15 minutes. The obtained solution was washed three to four times with equal volume of 0.7% normal saline solution and again centrifuged. Finally the RBC layer was collected and diluted to make 10% v/v suspension with normal saline.

Heat Induced Haemolysis [16-17]
A volume of 2.0mL of 10% RBC was added to 2.0mL of the extract. The resulting solution was heated at 56°C for 30 minutes then cooled to room temperature followed by centrifugation at 2500 rpm for 10 minutes. Supernatant was collected, and absorbance was measured at 560 nm. For control 2.0mL of 10% RBCs suspension and 2.0mL of 0.7 % Saline were added. Diclofenac sodium was taken as standard (positive control). Percent membrane stabilization was calculated by the method of Shinde *et al*. (1999); Saket *et al*. (2010) [19].

$$.\%\ \text{Inhibition} = \left(100 - \left(\frac{\text{ABSORBANCEsample} - \text{ABSORBANCEcontrol}}{\text{ABSORBANCEstandard}}\right) * 100\right)$$

Inhibition of Protein Denaturation [18-20]
A solution of 0.5% W/V of BSA was prepared in Tris buffer saline and pH was adjusted to 6.8 using hydrochloric acid. Test solutions consisted of 0.5mL each extract and 5.0mL of 0.5% W/V BSA. The control consists of 5.0mL 0.5% W/V BSA solution with 0.5mL methanol. The standard consist 0.5mL of Diclofenac Sodium in methanol with 5.0mL 0.5% W/V BSA solution. The sample extracts, standard and control were incubated at 37°C for 20 min and then heated to 51°C for 10 minutes. The reaction mixture was cool and the turbidity was measured spectrophotometrically at 660 nm. The % inhibition of precipitation (denaturation of the protein) was determined on a % basis relative to the control using the following formula.

RESULTS AND DISCUSSION

Phytochemical Screening:
Phytochemical screening shows methanolic extracts possess high amount of phytochemical presences in both parts of plants i.e. leaves and flowers. This shows why methanolic extracts have great potency for antioxidant and anti-inflammatory activities.

Table I: Phytochemical Testing Results: Leaves

CONSTITUENTS	P	T	C	E	M	W
Alkaloids						
i) Dragendorff's test	-	-	-	-	+	-
j) Hager's test	+	+	-	+	+	+
k) Wagner's test	+	-	-	-	-	-
l) Mayer's test	-	-	-	-	+	+
Flavonoids						
e) Shinoda test	-	-	-	-	+	-
f) Alkaline reagent NaOH test	+	-	-	+	+	+
Saponins						
c) Foam test	-	-	-	+	+	+
Carbohydrates						
c) Molisch" s test	-	-	-	-	-	-
Glycosides						
c) Legal" s test	+	+	-	-	-	-
Phytosterols & Triterpenes						
e) Libermann Burchard test	-	-	-	-	-	-
f) Salkowski test	+	+	-	+	-	+
Phenolic						
e) Ferric chloride test	+	-	+	-	+	-
f) Bromine water test	-	-	-	+	+	-
Tannins						
c) Lead acetate test	-	-	-	-	-	-

Table II: Phytochemical Testing Results: Flowers

CONSTITUENTS	P	T	C	E	M	W
Alkaloids						
m) Dragendorff's test	+	-	-	-	+	-
n) Hager's test	-	+	+	-	+	+
o) Wagner's test	-	-	-	+	-	-
p) Mayer's test	-	-	-	-	-	+
Flavonoids						
g) Shinoda test	-	-	-	-	+	-
h) Alkaline reagent NaOH test	+	+	-	-	+	+
Saponins						
d) Foam test	+	-	+	-	+	+
Carbohydrates						
d) Molisch" s test	-	-	-	-	-	-
Glycosides						
d) Legal" s test	+	-	-	-	+	-
Phytosterols & Triterpenes						
g) Libermann Burchard test	+	-	-	-	-	-
h) Salkowski test	+	-	+	-	+	-
Phenolic						
g) Ferric chloride test	-	-	-	+	+	+
h) Bromine water test	-	-	-	-	-	-
Tannins						
d) Lead acetate test	-	-	-	-	-	-

(+) = indicates presence, (-) = indicates absence.
P= Petroleum ether 60-80° C, T= Toluene, C= Chloroform, E= Ethyl acetate, M= Methanol, W= Water.

Table III: DPPH Assay: *Anisomeles malabarica* R.Br. Ex Sims

	% SCAVENGING ACTIVITY	
TEST SOLUTION	**LEAVES**	**FLOWERS**
WATER	42.63	35.41
MEOH	86.61	88.31
$CHCl_3$	84.92	79.39
ETAC	80.52	81.79
ESSENTIAL OIL	38.17	55.24
STANDARD AA	94.83	94.83

Table IV: Iron chelating Assay: *Anisomeles malabarica* R.Br. Ex Sims

	% CHELATING ACTIVITY	
TEST SOLUTION	**LEAVES**	**FLOWERS**
WATER	86.14	58.70
MEOH	87.70	91.91
$CHCl_3$	80.99	74.83
ETAC	84.29	89.01
ESSENTIAL OIL	74.35	67.72
STANDARD AA	95.84	95.84

Table V: Protein Inhibition Assay: *Anisomeles malabarica* R.Br. Ex Sims

	% INHIBITION	
TEST SOLUTION	**LEAVES**	**FLOWERS**
WATER	59.94	67.04
MEOH	89.19	87.06
$CHCl_3$	83.71	85.96
ETAC	84.08	85.17
ESSENTIAL OIL	69.52	67.72
STANDARD DFS	89.06	89.06

Table VI: Heat Induced Haemolytic Assay: *Anisomeles malabarica* R.Br. Ex Sims

	% MEMBRANE STABLIZATION	
TEST SOLUTION	**LEAVES**	**FLOWERS**
WATER	40.33	51.52
MEOH	79.72	94.87
$CHCl_3$	70.51	75.52
ETAC	64.33	89.63
ESSENTIAL OIL	56.64	65.27
STANDARD DFS	78.09	78.09

Antioxidant studies:

DPPH scavenging activity:

All plant different extract were measured by the ability to scavenge DPPH free radicals and was compared with the standard ascorbic acid. It was observed that methanol flower extract of the *Anisomeles malabarica* R.Br. Ex Sims shows higher activity than the leaves of the plant. The essential oil shows least scavenging activity (Table 3). This showed that the extracts have the proton donating ability and possibly act as primary antioxidants; could serve as free radical inhibitors or scavenging.

Iron chelating activity:

The reducing ability of the methanolic flower and leaves extracts for reduction of Ferric (III) to Ferrous (II) was found to be 91.91% and 87.70%. The standard ascorbic acid shows maximum 95.84% chelating activity (Table 4).

Anti-inflammatory studies

Inhibition of albumin denaturation:

The anti-inflammatory potential by membrane stabilization heat induced haemolytic was found to be high in methanol flower extract whereas leaves extracts showed high protein denaturation potency. These studies provide an indication for membrane stabilization and protein denaturation as an additional mechanism of *Anisomeles malabarica* R.Br. Ex Sims for anti inflammatory effect (Table 5 and 6).

CONCLUSION

Antioxidant and Anti- inflammatory potentials of different extract obtained from leaves and flowers of *Anisomeles malabarica* R.Br. Ex Sims were evaluated. The methanolic extracts were found to possess more radical scavenging activity, anti-inflammatory, antioxidant, as determined by protein denaturation, membrane stabilization, protease inhibition assay, scavenging effect on the DPPH, reducing power than the other solvent extracts. In general, results indicated that the extracts possess potent bioactivities.

In the present study, it is found that the methanolic extract of leaves and flowers of *Anisomeles malabarica* R.Br. Ex Sims contains a substantial amount of phenolics and flavonoids, and it is the presence of phenolics compounds may be responsible for their marked antioxidant activity. The result also shows a prominent binding of the ligand with anti-inflammatory targets. Thus, it can be concluded that methanolic extract of leaves and flowers of *Anisomeles malabarica* R.Br. Ex Sims can be used as an antioxidant, anti-inflammatory agent. Anti-inflammatory potentials of the selected extracts were depicted clearly in protein denaturation, membrane stabilization assay. The essential oil also shows some anti-oxidant and anti- inflammatory activities.

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