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IDENTIFICATION OF ANTIOXIDANT PRINCIPLE, RADICAL SCAVENGING AND ANTICANCER ACTIVITY OF INDIAN CAT'S WHISKER

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An HPLC method was developed for determination of the major antioxidant principle from the leaves of *Orthosiphon aristatus* Benth.from different parts of South India. Rosmarinic Acid, (RA) a caffeic acid derivative, was identified to be the major compound. The results showed significant variation in the amount of RA in methanolic extracts of leaf samples from different parts of South India. The potential antioxidative and anticancerous activities of ethanolic crude leaf extracts were evaluated *in vitro*. Antioxidative potency of the ethanol extracts was comparable to that of synthetic antioxidants BHT and tocopherol. The present observations also establish the efficacy of *Orthosiphon aristatus* leaves as potential anticancer agents.

Keywords : Antioxidant and anti cancer; HPLC; *Orthosiphon aristatus;* Rosmarinic acid.

Introduction

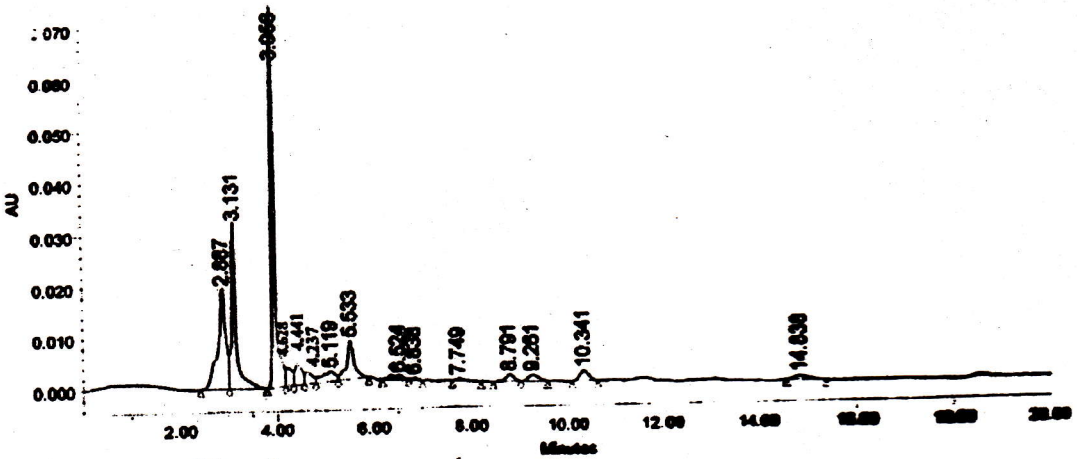
Due to pharmacological safety, there has been an increased interest in phytochemicals that may exhibit antioxidant activity which could be relevant in their nutritional incidence and their role in health and disease[1-3]. Antioxidants neutralize reactive oxygen which stress, diseases our cells and inflict damage to biomolecules, resulting in aging and genetic changes that lead to cancer. Common sources of antioxidants are fruits vegetables and medicinal plants. Therefore, a great number of different spices and aromatic herbs have been investigated for their antioxidant activity[4]. Some species particularly belonging to Lamiaceae, have been found to be very effective with regard to natural antioxidants. In various studies, *rosemary, sage, oregano,* and *thyme* have shown strong antioxidant activity[5-8]. Currently,there has been a considerable interest in finding naturally occurring antioxidants to replace synthetic antioxidants in foods and medicinals[9]. Several studies have analysed the antioxidant potential of a variety of herbs[10,11]. Among the different parts of plants studied, the leaves are reported to have highest antioxidant properties[12,13] and the most active principle among the phytochemicals is the phenolic fraction[14,15]. The phenolics have *in vivo* antioxidant activities and have been used as natural antioxidants in food[16].

Orthosiphon aristatus, a member of the Lamiaceae family,is a popular medicinal herb in South-East Asia. The plant has proven properties to exert anti-diabetic and lipid lowering effect in diabetic rats[17]. In Malaysia, *Orthosiphon aristatus* is traditionally used to promote urination and to alleviate bladder and kidney discomfort[18]. The therapeutic effects of *Orthosiphon aristatus* have been ascribed mainly to its polyphenols, which have enzyme inhibition and antioxidant activity[19].

Though considerable work have been reported in *Orthosiphon aristatus,* a comparative study has so far not been done in India. The aim of this work is to identify the major antioxidant principle in leaves of *Orthosiphon aristatus*,and to compare its variation in the accessions from different regions of South India, as well as to evaluate their antioxidative and anticancerous properties. A rapid HPLC finger printing of the samples was achieved by reverse phase isocratic elution with potassium hydrogen phosphate buffer: acetonitrile with UV detection for the identification of the marker.

Material and Methods

Eagle's minium essential medium and trypsin were obtained from Hi Media India Rosmarinic acid was purchased from Sigma Chemicals. Solvents used for HPLC were potassium dihydrogenphosphate and acetonitrile were obtained from Merck. Membrane filters from Millipore were used for filtration of the mobile phase and the samples. All other chemicals and reagents used were of analytical reagent quality.



Chromatogram of the reference compound

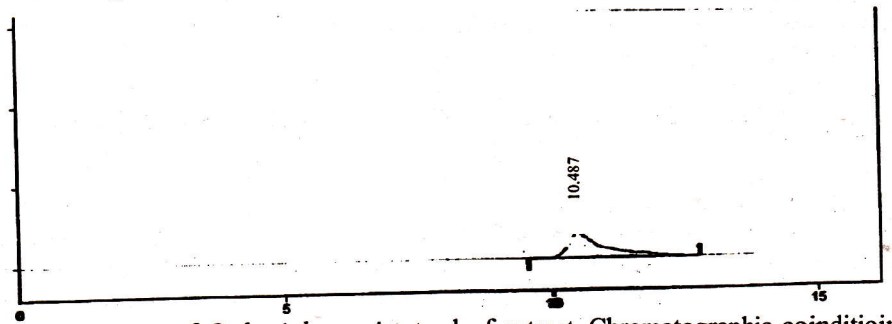


Fig.1. Typical chromatogram of *Orthosiphon aristatus* leaf extract. Chromatographic coinditioins : 7725 Rheodyne injector, Waters 510 HPLC pump, 486 tunable absorbance detector, eluent potassium dihydrogen phosphate : acetonitrile (75:25); flow rate of 0.8ml/min; UV detection at 254nm and the injection volume was 10µl.

Plant Materials-The leaves of the plant, used for the study were collected from different parts of South India. Voucher specimens are deposited in the Herbarium of the Department of Botany, University of Kerala. The collected leaves were washed, chopped into pieces and air dried. Fifty gram of the powdered plant portion was filled in a soxhlet extractor and extracted with 300ml ethanol (Boiling point - 64 to 65°C) for ten hours. The extract was transferred to a conical flask, concentrated and evaporated to dryness in a vacuum rotary evaporator under reduced pressure[20] and used for further analysis of antioxidant and anticancer properties.

HPLC analyses

a) Preparation of samples-The samples were prepared following the method of Beta *et al.*[21]. One gm of fresh leaf tissue was finely chopped and put in boiling 80% methanol and refluxed for 10 min. The extract was cooled and the tissue was homogenized with a pestle for a few minutes. The homogenate was filtered and centrifuged for 10 min at 10000 g. The supernatant was taken for the estimation of phenolic compounds.

b) Identification and Quantification of major antioxidant principle in the leaf sample by RP-HPLC- Phenolic constituents of the extract from the leaf tissues were fractionated by RP-HPLC. An HPLC system [Waters] equipped with a 7725 Rheodyne injector, Waters 510 HPLC pump, 486 tunable absorbance detector and Millenium 2010 software data module were used for the study. An HPLC column of 4.6 x 250 mm id reverse phase [RP] C18 was used for the fractionation of phenolic acids. Potassium hydrogen phosphate buffer: acetonitrile was used as the mobile phase in the ratio 75:25. An elution period of 20 min with a flow rate of 0.8 ml /min was given with a 10 µl injection volume and the absorbance at 254 nm was recorded. Marker compound in the sample was identified with the retention time of the standard and area of the peaks was taken for quantification. Concentration of the standard and area of the standard peak were taken as standard parameters.

Determination of antioxidant activities

DPPH radical scavenging activity-The free radical scavenging activity of the extract was determined using

DPPH. stable radical following the methodology described by Blois[22]. Briefly 0.1mM solution of DPPH. in methanol was prepared and 1ml of this solution was added to 3ml of the plant extract in methanol at different concentrations (100-1000µg/ml). Thirty minutes later, the absorbance was measured at 517nm. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

Hydrogen peroxide scavenging assay-Hydrogen peroxide scavenging ability of the plant extract was determined according to the method of Ruch *et al.*[23]. Hydrogen peroxide (40mM) solution was prepared with standard phosphate buffer [pH 7.4]. Different concentrations of the fractions [100-1000 µg/ml] in distilled water were added to 0.6 ml H_2O_2 solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing the phosphate buffer without hydrogen peroxide.

Phosphomolybdate method- The total antioxidant capacity of the fractions was determined by phosphomolybdate method[24] using α- tocopherol as the standard. An aliquot of 0.1 ml of the fractions [100 µg] was combined with 1 ml of reagent [0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate]. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against the blank using UV spectrophotometer. The blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as microgram equivalents of α- tocopherol by using the standard graph.

Statistical analysis - All data on antioxidant activity are the average of triplicate analysis. The data were recorded as mean±SD and analyzed by SPSS (version 10 for Windows 98, SPSS Inc). One way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests. Values $p< 0.05$ are regarded as significant.

Determination of in vitro cytotoxic effect of the crude extracts - The short time *in vitro* cytotoxicity studies were done by trypan blue exclusion method using DLA cells.

MTT assay - The *in vitro* response of the crude methanolic fraction of the leaves of *Orthosiphon* against HeLa, HCT, B16F10, MCF7, and C33A cell lines was studied using MTT assay. Briefly cells were seeded at a density of $3x10^4$ cells/wells into 24 well plates. After 24h the extract was added to the medium at various concentrations and incubated at 24 or 48 hrs as indicated. At the end of incubation, 50µl of 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (2mg/ml) per well was added and the formazan crystals formed were solubilized in acidified isopropanol after aspirating the medium. The extent of MTT reduction was measured spectrophotometrically at 570nm, and the cell survival was expressed as percentage over the untreated control.

Results and Discussion

Concentration of the major antioxidant principle in methanolic leaf extracts of Orthosiphon aristatus. - In the present study, an isocratic method was used for the identification and quantification of RA, which was separated in a total time of 20min. The standard was resolved and eluted at 10.32 min. The marker showed a good linearity in the range from 2.0 to 1000ng in the caliberation curves that was obtained by HPLC analysis. The reference marker was present in the chromatographic profiles from various locations when the sample solution was analysed by HPLC. The peak of RA was confirmed by comparison of the retention time with the reference standard (Fig.1.).

The HPLC procedure was applied to the determination of the marker compound in OS samples from different regions of south India. All the analysed samples showed a wide range in the concentrations of RA, which may be ascribed to environmental conditions and variation in sample sourcing. The values obtained for the marker appear to fall within the range reported for the markers in *Orthosiphon aristatus* leaves[25]. However, the overall levels of the marker concentration were markedly higher in samples from Wayanad. Concentration of RA varied from 12.3% to 10.6% of total fresh leaf weight (Table 1). The HPLC results showed that the relative concentrations of the markers varied considerably. Based on these observations an evaluation was done on the antioxidant and anticancer activity of the extract which showed highest concentration RA.

Antioxidant activity - The present study assumes relevance on the beneficial effects of the alcoholic leaf extract of *Orthosiphon aristatus* on free radical scavenging. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or by protecting the antioxidant defence mechanisms. The antioxidant activity of the plant extract may be due to the phenolic compounds, which is considered as a powerful free radical scavenger[26]. Phenolic compounds on encountering free radicals with unpaired

Table 1. Percent concentrations of RA in leaf samples of *Orthosiphon aristatus* collected from different regions of South India.

Location	Concentration of RA (% of Total fresh leaf weight)
Wayanad	12.3%
Palode	10.6%
Malappuram	11.3%
Maruthwamala	11%
Nagercoil	11.6%

Table 2. Free radical scavenging activity of the extract and the standards.

	Fractions	IC_{50} (mg/ml)
DPPH	EOS	0.980
	BHT	1.60
OH	EOS	0.422
	BHT	0.642
H_2O_2	EOS	1.16
	Tocopherol	0.566

Table 3. The cell viability assayed by MTT. Percentage of cell viability.

Drug	Conc. µg/ml	Cell lines used				
		HeLa	HCT	B16F10	MCF7	C33A
Alcoholic plant extract (EOS)	10	77	89	93	88	88
	25	75	91	92	97	87
	50	67	94	97	96	75
	100	51	92	96	95	73

electrons, supplies an equal and opposite negative charge to neutralize the bad effects of free radicals. The tests used to evaluate the potency of the extracts as antioxidants are well established model systems; based on radical scavenging ability of the leaf extract. Flavanoids and phenolic acids have been implicated as natural antioxidants in plants, fruits and vegetables[9]. DPPH assay is one of the most widely used methods for screening antioxidant activity of the plant extracts[27]. DPPH is a stable, nitrogen-centered free radical, which produces violet colour in ethanol solution. It was reduced to yellow coloured product, diphenylpicrylhydrazine, with the addition of the fractions in a concentration dependent manner. The reduction in the number of DPPH molecules can be co related with the number of available hydroxyl groups. The plant extract showed significantly higher inhibition percentage (stronger hydrogen- donating ability) and may positively correlate with phenolic components. The DPPH radical scavenging activity of the extract (IC_{50} 0.980mg/ml)was significantly greater when compared to that of the standard BHT (IC_{50} 1.60mg/ml). Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde, which produces a pink chromogen with thiobarbituric acid[28]. Alcoholic plant extract when added to the reaction mixture, scavenged the hydroxyl radicals and prevented the degradation of deoxyribose. The activity was comparable to that of the standard. The plant extract and the standard BHT inhibited the production of hydroxyl radicals. The scavenging activity of *Orthosiphon aristatus* extract (IC_{50} 0.422mg/ml) was higher than that of BHT (IC_{50} 0.642mg/ml). H_2O_2 itself is not very reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals, which is very toxic to the cell[28]. Thus scavenging the H_2O_2 is a measure of the antioxidant activity of the fractions. The plant exract fractions scavenged hydrogen peroxide, which may be attributed to the presence of phenolic compounds that could donate electrons to hydrogen peroxide, there by neutralizing into water. However the scavenging activity of the plant (IC_{50} 1.16mg/ml) was significantly less compared to standard α tocopherol.(IC_{50} 0.566mg/ml). The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of the extracts[29]. In the presence of the fractions, Mo (VI) is reduced to Mo (V) and forms a

green coloured phosphomolybdenum V complex, which shows maximum absorbance at 695nm. The plant extract showed a very high activity when compared to the standard. The method is quantitative, since the total antioxidant capacity is expressed as α-tocopherol equivalent. The extract contains 12.77µg α-tocopherol equivalents/mg. Based on the results obtained it may be concluded that ethanolic extract of *Orthosiphon aristatus* has strong antioxidant and free radical scavenging activities when compared to the standards such as α tocopherol, and BHT(Table 2). The presence of phenolic compounds may be responsible for the activity.

Anticancer activity -Pharmacologically safe compounds that can inhibit the proliferation of tumor cells have potential as anticancer agents. Most of the drug used today for cancer therapy act by their cytotoxic effect but with a mediocre specificity and hence a high secondary toxicity. Cytotoxic screening models provide important preliminary data to select plant extracts with antineoplastic properties. The cell viability assayed by MTT decreased with increase in concentration. For each cell line, the concentration required to reduce absorbance by 50% in comparison to control cells was determined (Table 3) and extract showed good inhibition against HeLa cell line with an IC_{50} at 100 µg/ml.

The HPLC chromatographic profiles of the *Orthosiphon aristatus* samples from different locations were qualitatively similar but the results show variations in the concentrations of the phenolic compounds. In the past few years there has been increasing interest in finding natural antioxidants because they can protect the human body from free radicals and ROS related effects and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in food. According to data obtained from the present study the leaf extract exhibited different but effective degrees of *in vitro* antioxidant activity, with all the methods used, when compared to standard antioxidant compounds. The HPLC finger printing could be used in authentication of *Orthosiphon aristatus* samples and formulations. Further studies in *in vivo* antioxidant activities and evaluation of the molecular mechanism that underlie the modulation of antioxidant activity are being investigated in our laboratory.

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