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Research Article

SCREENING BIOLOGICAL ACTIVITIES OF ORTHOSIPHON ARISTATUS

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ABSTRACT

One of the prerequisites for the success of primary health care is the availability and use of suitable drugs. Plants have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. It is thus reasonable for decision-makers to identify locally available plants or plant extracts that could usefully be added to the national list of drugs, or that could even replace some pharmaceutical preparations that need to be purchased and imported.

Orthosiphon aristatus Benth. [Lamiaceae] is an important plant in traditional folk medicine. The plant has extensively been exploited traditionally to treat several human ailments. The main objective of the study was the pharmacological evaluation of *Orthosiphon aristatus,* which is widely used in the management of various diseases, inorder to assess its efficiency claim and substantiate its pharmacological applications. *In – vitro* experiments were carried out inrder to investigate the antioxidant, anticancer, anti bacterial and anti-inflammatory activities using ethanolic plant extract. The result show less anti bacterial, anti cancer and anti inflammatory activities than the respective standards. The best results were obtained with antioxidant study. It was found that *O. aristatus* exhibit antioxidant activities, which make the plant an attractive subject for further experimental and clinical investigations. The ability of the extract to scavenge the free radical is an indication of the broad spectrum antioxidant potential of *O.aristatus*, which make the plant a candidate for bio prospecting for antioxidant drugs

Keywords: DPPH, free radical, hyalurodinase enzyme, disc diffusion, 3-[4-5 dimethylthiozol-2-yl], 2-5 diphenyl-tetrazolium bromide, dimethyl sulphoxide, IC_{50} values.

INTRODUCTION

People in all continents have long applied poultices and imbibed infusions of hundreds, if not thousands, of indigenous plants, dating back to prehistory [1].The plant chemicals used for these purposes are largely the secondary metabolites which are derived biosynthetically from plant primary metabolites and are not directly involved in the growth, development or reproduction of plants. Plants have been described as chemical factories that are capable of synthesizing unlimited numbers of highly complex and unusual chemical substances whose structures could escape the imagination of synthetic chemists forever. A single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as antioxidants and venotonics, anti-bacterial and anti-fungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being[2].In short, botanical drugs could provide an unique opportunity to bio prospect diverse and synergistic chemical moieties, which in combination might act on multiple targets and improve the therapeutic spectrum. Traditional knowledge and practices bring experimental wisdom to provide a safer and more cost effective platform for newer scaffolds and drug discovery.

Although a great potential exists for discovering new drugs from natural product chemistry, the methods in drug screening and evaluation are perhaps still in their infancy. The screening of natural products usually involves stepwise methodical procedures of [a] detecting active chemical substances in crude extracts from a diversity of species collections, [b] isolating chemical agents from samples through fractionation guided by activity in bioassay systems, and [c] chemical and pharmacological evaluation of isolated active compounds employing correlative [accessory] bioassays that have predictability in drug development; the term screening refers to any one step or any combination of steps, collectively. [3; 4; 5; 6; 7; 8].

Screening active compounds from plants have lead to the discovery of new medicinal drugs which have efficient protection and treatment roles against various diseases [9; 10; 11; 12; 13]. Many of the phytochemical bioactive compounds from medicinal plants have shown many pharmacological activities [14; 15; 16; 17].

There is a great need for standardization of herbal medicine using modern scientific and multidisciplinary approach [18; 19]. This would pave ways for making available medicinal preparations of reliable quality which even a common man can afford. Pharmacological evaluation of *Orthosiphon aristatus*, which is widely used in the management of various diseases, was carried out in order to assess its efficiency claim and substantiate its pharmacological applications.

MATERIALS AND METHODS

Plant material

Leaves of *Orthosiphon aristatus* Benth, used for pharmacological screening, belonging to Lamiaceae family, were obtained from collections of medicinal plants maintained in the Garden of Department of Botany, University of Kerala. The collected leaves were washed, chopped into pieces and air dried in the shade, at room temperature [25^{0}C] for ten days.

Preparation of plant extract

Air dried leaf samples were powdered separately and used for extraction. Fifty gram of the powdered plant portion was filled in a soxhlet extractor and extracted with 300ml ethanol [boiling point - 64 to 65^{0}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].

Screening for antioxidant activity using DPPH free radical scavenging assay

The free radical scavenging activity of the extract was determined using DPPH stable radical following the methodology described by Molyneux[21]. 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 [5-250µg/ml]. Thirty minutes later, the absorbance was measured at 517nm. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

Screening for antibacterial activity using disc diffusion method

The ethanolic plant extracts were tested against microorganisms namely *Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 741, *Staphylo-coccus aureus* MTCC 740, and *Bacillus*

subtilis MTCC 10619 in order to determine the antibacterial activity.

Disc diffusion method for detection of antibacterial activity

Sensitivity tests were performed by disc diffusion method which is a modified form of Kirby-Bauer test [22]. Nutrient agar was melted in hot water bath at 90˚c and allowed to cool. When the temperature reached 50˚c, it was poured to sterile petri dishes under laminar air flow chamber and allowed to solidify in a horizontal position. The culture colonies from the stock cultures were selected and 100µl was transferred to the culture medium. A sterile spreader was dipped into the properly diluted inoculums and spread evenly.

Sterile filter paper disc of diameter 5mm [made from Whatman No-1 filter paper] impregnated with extract [100 µg/ml] prepared in 10% DMSO were placed on the surface of the nutrient agar plates seeded with test organisms. They were touched down with a sterile forceps to ensure complete contact with the surface. Then the plates were incubated at 37˚c for about 24 hrs. Control disc impregnated with dimethyl sulphoxide [DMSO] and reference drug [streptomycin - 10µg/ml] were also used along with the test disc in each experiment. The plates were examined after 24hrs incubation. The zone showing incubation was determined by gross virtual examination and the diameter of the zone to the nearest mm was recorded with a ruler.

MTT assay for determination of anti-cancer activity

The in vitro response of the crude ethanolic fraction of the leaves of *Orthosiphon aristatus* against five different cell lines, cervical-HeLa, colon-HCT116, mouse melanoma-B16F10, breast-MCF7 and Hepatic G2- C3A, was studied using MTT assay. Briefly the cell lines were seeded at a density of $3x10^4$ cells/wells into 24 well plates. After 24hrs, 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.

Hyaluronidase inhibition assay for determination of anti-inflammatory activity

The assay was performed according to Ling *et al*. [23]. The assay medium consisted of 3,5 U-hyaluronidase in 100µl 20mM sodium phosphate buffer pH 7.0 with 77mM Sodium Chloride, 0.01% BSA pre incubated with different concentrations of the test compound [in DMSO] for 15 min at 37˚c. The assay was commenced by adding 100µl hyaluronic acid [0.03% in 300mM sodium phosphate, pH 5.35] to the incubation mixture and incubated for a further 45min at 37˚c. The undigested hyaluronic acid was precipitated with 1ml acid albumin solution made up of 0.1% bovine serum albumin in 24mM sodium acetate and 79mM acetic acid[pH3.75]. After keeping the reaction mixture at room temperature for 10 min, the absorbance was measured at600nm. The absorbance in the absence of the enzyme was used as the reference value for maximum inhibition. The inhibitory activity of the test compound was calculated as the percentage ratio of the absorbance in the presence of test compounds vs absorbance in the absence of the enzyme. The enzyme activity was checked by control experiment run simultaneously, in which the enzyme was preincubated with 5µl DMSO instead, and followed by the assay procedures described above. Compounds were tested in a range of 5µg - 250 µg in the reaction mixture. Indomethacin was used as reference standard.

Statistical analysis

Results were analyzed using One way Analysis of Variance [ANOVA] and expressed as mean ± SE. Data was further subjected to Duncan's post hoc analysis and differences between means were regarded significant at $P<0.05$.

RESULTS

Anticancer activity

In this study, five tumorogenic cell lines including, HeLa, HCT, B16F10, MCF7 and C3A were chosen to determine the anticancer activity of *Orthosiphon aristatus* extracts. Cultures of HeLa, HCT, B16F10, MCF7 and C3A were treated with increasing concentrations [5 - 100µg] of the ethanolic extracts of *O. aristatus* [Fig 2.1a - Fig 2.1e]. As shown in the figure, for HeLa cells, the cell growth inhibition was 67% in the presence of plant extract at 50µg/ml and the activity of this extract reached an IC_{50} value at 100µg/ml. On HCT, B16F10, MCF7 and C3A the plant extract exhibited no activity. All the cell lines tested showed high IC_{50} values.

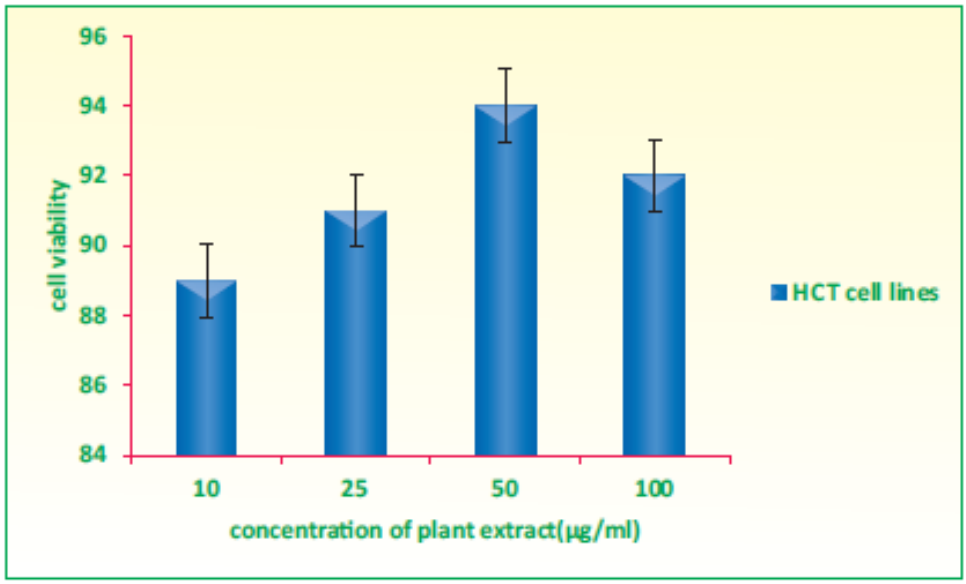

Fig. 2.1a: Effect of ethanolic extract of *O.aristatus* on HCT cell lines. Data are presented as the mean SE of results from four independent experiments and the error bars indicate standard error. The difference among the mean values were analysed using one way ANOVA followed by Duncan post-hoc analysis.

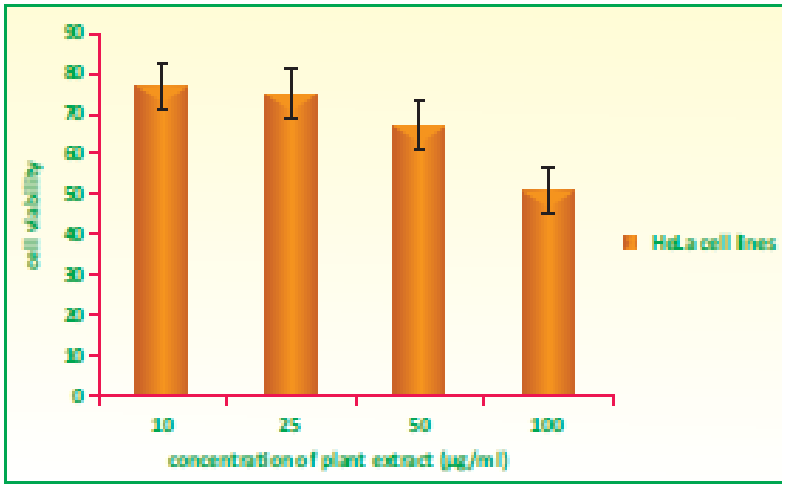

Fig. 2.1b: Anticancer activity of ethanolic extract of *O.aristatus* against HeLa cell lines. The cell viability was measured on quadruplicate by MTT assay. The results are expressed as the mean percentage of quadruplicate determinations. The differences among the mean values were analysed using one way ANOVA followed by Duncan post-hoc analysis.

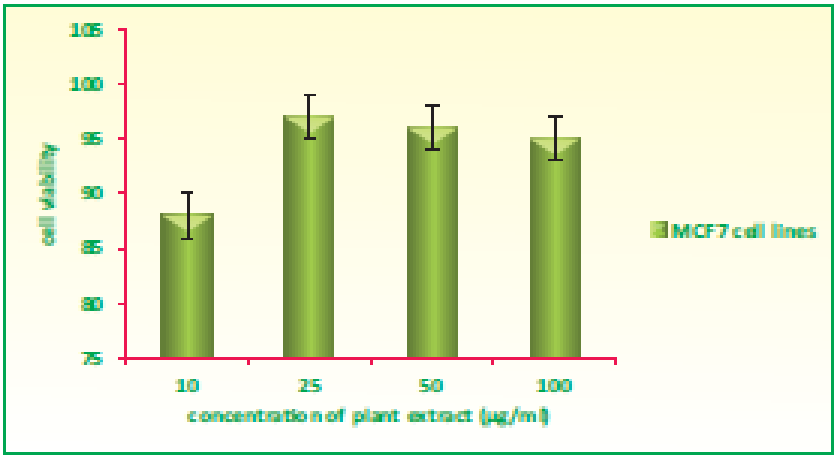

Fig. 2.1c: Anticancer activity of ethanolic extract of *O.aristatus* against MCF7 cancer cell lines. The cell viability was measured on quadruplicate by MTT assay. The results are expressed as the mean percentage of quadruplicate determinations. The differences among the mean values were analysed using one way ANOVA followed by Duncan post-hoc analysis.

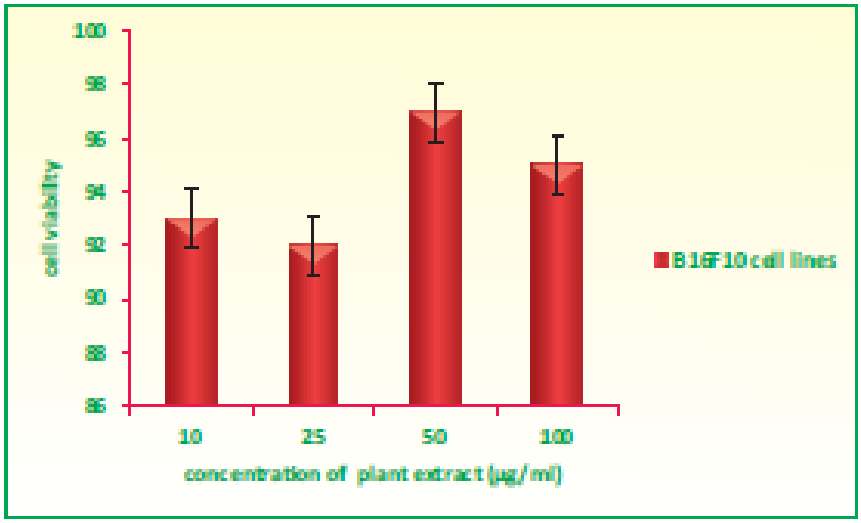

Fig. 2.1d: Shows cell viability on B16F10 cancer cell line at different concentrations by the ethanolic plant extract of *O.aristatus.*results are the mean ± SE of 4 replicates. The differences among the mean values were analysed using one way ANOVA followed by Duncan post-hoc analysis. The error bars indicate standard error.

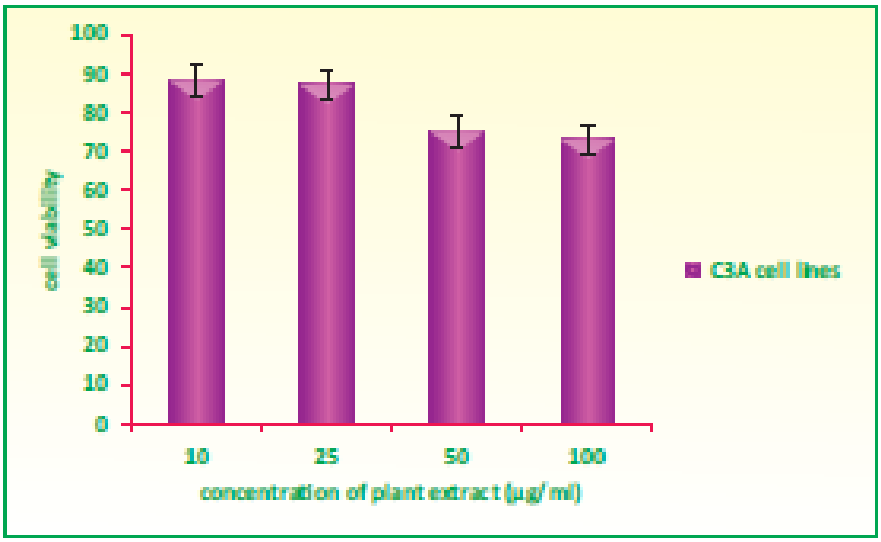

Fig. 2.1e: Shows cell viability following treatment with ethanolic plant extract of *O.aristatus* on C3A cell line as assessed using MTT assay. Data is the mean ± SE for 4 replicates. The differences among the mean values were analysed using one way ANOVA followed by Duncan post-hoc analysis.

Antibacterial activity

The crude extract of *Orthosiphon aristatus* was subjected to antibacterial activity against the five pathogenic bacterial strains using disc diffusion method. The antimicrobial activity of the extracts and their potency was quantitatively assessed by the presence / absence of inhibition zone and zone diameter, respectively. The diameter of zone of inhibition was different for each concentration and for each bacterium [table-2.1]. The extracts showed a good activity against the gram positive bacteria *S. aureus* and *B. subtilis* with a zone diameter of 11 mm, a moderate activity on the gram negative *P. aeruginosa* with an inhibition value of 7mm and a weak activity against the gram negative *E.coli*. Results are given in table 2.1.

Lower concentrations of the extract, when used, failed to produce inhibition zones. The relative extend of susceptibility of the tested organisms to the plant extract followed a decreasing order of *S. aureus*, B.*subtilis, P.aeruginosa* and *E.coli*. In other words *S. aureus* was the most susceptible organism while E.coli was the most resistant microbe studied. The results revealed variability in the inhibitory concentration of extract for each given bacterium.

Table 2.1: Inhibition zone indicating the antibacterial activity of extracts of *O.aristatus*

	Concentration of plant extract used (µg/ml)					Streptomycin
	10	25	50	100	250	
S.aureus	-	+	+	++	+++	+++
B.subtilis	-	-	+	++	+++	+++
E.coli	-	-	-	-	+	+++
P.aeruginosa	-	-	+	+	+	+++

(-): no inhibition; (+): weak inhibition (<8mm): ++ modest inhibition (8mm-10mm); +++ strong inhibition (≥ 10 mm); all readings were inclusive of 5mm disc diameter.

Antioxidant activity

The change in colour of DPPH from deep violet to yellow is observed in all the concentrations used which is an indication of antioxidant activity. Fig 2.2 shows the decrease in the concentration of DPPH radical due to the scavenging ability of the ethanolic plant extracts and the commercial standards. In this study we used BHA and BHT as commercial standards. In the present work, the plant extract at 100µg/ml showed an excellent DPPH radical scavenging activity [42.25%] which was greater than that of the commercial standard used and the activity increased with increasing concentrations [Fig 2.2]. Under same experimental conditions, positive control counter parts, BHA and BHT at 100µg/ml showed 40.25% and 55% DPPH radical scavenging activities, respectively. The 50% inhibition of DPPH radical by test samples [IC_{50}] was observed at a concentration of 125µg/ml and at the same dosage there was a lower free radical scavenging activity for BHA and a higher activity for BHT, both of which are well known antioxidant compounds.

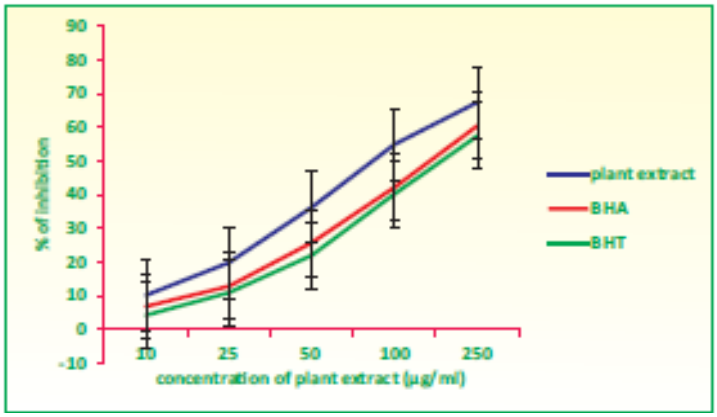

Fig. 2.2: DPPH radical scavenging activities of ethanolic extract of *O.aristatus* and the reference compounds BHA and BHT. The data represent the percentage inhibition of DPPH radical. The results are mean ± SD of four parallel measurements.

Antiinflammatory activity

In the present study, the anti inflammatory activity of *O.aristatus* was assessed by hyaluronidase assay. Indomethacin was used as the positive control which showed significant [P<0.05] anti inflammatory activity in reducing hyaluronidase enzyme. Extractof *O.aristatus* did not show significant activity in reducing the enzyme, instead showed a trend of anti inflammatory activity [p<0.05] [Fig 2.3]. Treatment with the plant extract showed an IC_{50} value more than 250µl.

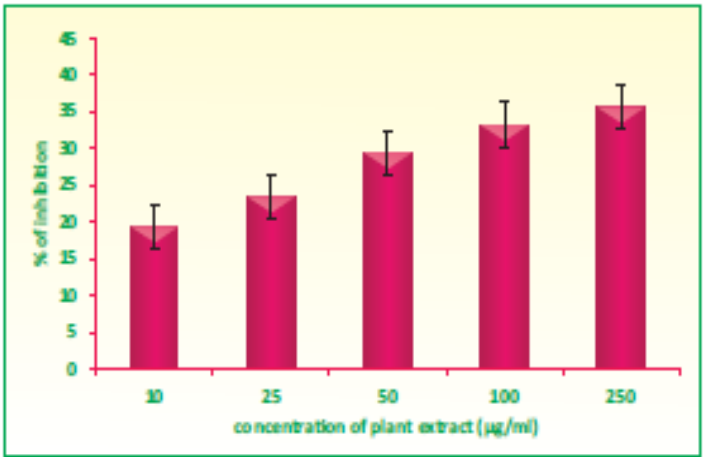

Fig. 2.3: Data expressed as effect of crude plant extract on hyaluronidase enzyme mean ± SE, n=4 (Anova post-test Duncan; P<0.05)

DISCUSSION

Plants have become the focus of increasing attention from many research groups in recent years, owing to their exciting chemistry and their wide spectrum of pharmacological activities. Pharmacological screening is performed to allow targeted isolation of new or useful constituents with potential activities. Epidemiological studies over the last three decades have consistently correlated certain diets, specific foods and disease expressions. At the same time, number of bioactive compounds has increased dramatically and a new diet-health paradigm has evolved that emphasizes the positive aspects of diet.The terms"phytochemical", "nutraceutical" and "functional food" have been introduced to describe various aspects of this development [24].

Ethanolic extracts of *Orthosiphon aristatus*, were evaluated for their potential anticancer, antibacterial, anti-inflammatory and antiradical effects, in the present work.

Successful determination of biologically active compounds from plant materials is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants [25]. The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay [26]. The choice will also depend on the targeted compounds to be extracted [27; 28]. Ethanol is more efficient in cell wall and seed degradations which have non-polar character and cause polyphenols to be released from cells. More useful explanation for the decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive.

Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material [29]. Since nearly all of the identified bioactive components from plants are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction [30]. Methanol is more polar than ethanol but due to its cytotoxic

nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.

The majority of most useful and curative anticancer drugs continue to be derived from plant sources. Doubtlessly, anticancer drugs of biological origin will continue to be of great importance in improving cancer treatment and overall survival rates. Plant extracts that contain several pluripharmacological compounds have been reported to act on multiple molecular and cellular targets and such approach is gaining support to fight cancer. The MTT cell viability assay is widely used in determining drug sensitivity in primary screening of potential chemotherapeutic drug. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The extract of *O.aristatus* showed moderate activity against the different cell lines tested. The different concentrations of extract did not appreciably alter the rate of apoptosis which act as a prelude to cell destruction.

A compound is judged as a good anticancer drug, when it is more specific towards tumour cells, in comparison to normal cells. Induction of apoptosis in cancer cells or malignant tissues is recognized as an efficient strategy for cancer chemotherapy. A number of antitumour agents including bleomycin, camptothecin, doxorubicin and etoposide have been shown to induce cell death by apoptosis [31; 32]. In the US NCI plant screening program, a crude extract is generally considered to have in vitro cytotoxic activity if the IC_{50} value [concentration that causes a 50% cell kill] in carcinoma cells, following incubation between 48 and 72 hours, is less than 20µg/ml, while it is less than 4 µg/ml for pure compounds [33].

Disk diffusion methods are extensively used to investigate the anti-bacterial activity of natural antimicrobial substances and plant extracts. These assays are based on the use of disks as reservoirs containing the solution of substances to be examined. The antimicrobial activity of the extracts and their potency was quantitatively assessed by the presence / absence of inhibition zone and zone diameter, respectively as shown in table 2.1. According to the disc diffusion method for antimicrobial activity, a prominent antibacterial effect, worthy of further investigation was not obtained for all the strains tested. The zones of inhibition by the extracts were compared with the zone of inhibition of reference drug streptomycin. The inhibition zones produced by all the extracts were found to be less than the inhibition zone of streptomycin. Absence of activity was found at lower concentrations when tested against *E.coli*. The activity was more pronounced against gram positive than against gram negative bacteria. The reason for the different sensitivity between gram-positive and gram-negative bacteria could be ascribed to the morphological differences between these micro-organisms, gram-negative bacteria having an outer phospholipid membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da [34].The gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not a selective permeability barrier [35].

Functional foods and beverages are typically developed with specific health goals such as reducing inflammation. Medicinal plants have shown great promise in the management of various inflammatory disorders and have continued to serve as alternative and complementary therapies. One enzyme that is involved in tissue remodeling during inflammation is hyaluronidase, which degrades glycosamino-glycans, including hyaluranan, in human and animal tissues. Hyaluranan polymers are important constituents of the extracellular matrix of connective tissues, including cartilage, the synovial membrane and synovial fluid joints. Many plant derived poyphenolics exert effects on hyaluronidase and other enzymes regulating extracellular matrix metabolism. In this work, the anti - hyaluronidase activity of *O. aristatus* was tested invitro in comparison to the widely used indomethacin. The data indicate an inhibition of 19.38, 23.45, 29.44, 33.22 and 35.62% of anti-inflammatory activity when treated with10, 25, 50, 100 and 250µg/ml of plant extract respectively. The positive control, indomethacin produced 54.4% inhibition at a concentration of 10µg/ml. Based on the results, it can be concluded that the plant extract cannot be considered as an effective and safer alternative to non -steroidal anti inflammatory drugs.

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. Since the DPPH assay can accommodate a large number of sample in a short period and is sensitive enough to detect natural compounds at low concentration, it was used in the present study for a primary screening of the free radical scavenging activity of the crude extract [36;37;38]. In the free radical scavenging assay, the effect of the plant extract was equivalent or slightly higher than those of commercial counter parts at the sameconcentration. All samples showed a dose dependent manner in scavenging DPPH radical that was statistically significant [$p<0.05$] when compared with control. The result demonstrates that *O. aristatus* extract has an inhibitory effect on the DPPH radical. In addition, the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation [39; 40; 41]. Thus the plant can be considered as an easily accessible source of natural antioxidant and can be considered as a suitable candidate for antioxidant drug discovery.

CONCLUSION

The use of traditional medicine is expanding to newer horizons and plants still remain as the novel source of structurally important compounds that lead to the development of innovative drugs. It is anticipated that plants can provide potential bioactive compounds for the development of new 'leads' to combat various diseases. The systematically performed in vitro assays revealed that the tested plant extract may find in therapy as agent with high pharmaceutical value. The results show less antibacterial, anti inflammatory and anti cancer activity than the respective standards. Considering anticancer activity, the extract showed high IC_{50} values for all the cell lines tested. The data on anti-inflammatory activity indicate an inhibition percentage of 19.38, when treated with 10µg/ml of extract while indomethacin produced 54.4% inhibition. For antibacterial activity, inhibitory values at higher concentrations were quite good. In general, more activity was obtained when using more concentrated decoctions of the plant extract studied. The best results [60.5% inhibition] were obtained with antioxidant activity. This study provides evidence that the plant extract have antioxidant properties, as tested through the DPPH method. Therefore, the plant may have great relevance in the prevention and therapies of diseases in which oxidant or free radicals are implicated. The ability of the extracts to scavenge the free radical is an indication of the broad spectrum anti oxidant potential of *Orthosiphon aristatus*, which make the plant a candidate for bio prospecting for antioxidant drugs. In addition, the plant is a good candidate for further phytochemical and chromatographic studies to isolate and fully characterize the compound related to this in vitro biological activity.

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