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Qualitative and quantitative phytochemical screening and in vitro anti oxidant and anti microbial activities of *Elephantopus scaber Linn.*

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Abstract

Preliminary phytochemical analysis and quantification of total phenols, *In-vitro* antioxidant and anti microbial activities of the different fractions (hydro alcoholic, hexane, ethyl acetate and methanol) of *Elephantopus scaber* were carried out against five selected pathogenic bacteria and three fungal species. The plant fraction possesses steroids, triterpinoids, saponins, flavonoides, carbohydrates, glycosides and oils. For total phenolic content gallic acid was taken as a standard, the ethyl acetate fraction contains rich phenolic content than other fractions and the methanol fraction shows more DPPH, superoxide and hydroxyl radical scavenging activity. In Anti-microbial activity study all fractions showed good inhibition zone against three organisms *i.e*., *Escherichia coli, Staphylococcus aureus Klebsiella pneumonia* among the other test organisms along with *Candida spp (fungal organism).*

Keywords: *Elephantopus scaber,* total phenols, antioxidant activity, DPPH radical, super oxide radical, hydroxyl radical, Anti infectious Activity, Osteomyelitis.

INTRODUCTION

India is a treasure chest of biodiversity which hosts a large variety of plants and has been identified as one of the eight important 'Vavilovian' centres of origin and crop diversity. A good proportion of species of Medicinal Plants do occur throughout India. Medicinal plants which constitute a segment of the flora provide raw material for use in all the indigenous systems of medicine in India namely Ayurveda, Unani, Siddha and Tibetan Medicine. According to the World Health Organization (WHO), 80% of the population in developing countries relies on traditional medicine, mostly in the form of plant drugs for their health care needs. Additionally, modern medicines contain plant derivatives to the extent of about 25%.

On account of the fact that the derivatives of medicinal plants are non-narcotic having no side effects, the demand for these plants is on the increase in both developing and developed countries. After information technology, herbal technology is India's biggest revenue source according to Sharma *et al,* 2008[1].

Many plant species display antioxidant and anti infectious arthritis properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus it is important to characterize different types of medicinal plants for their antioxidant and anti infectious arthritis potential according to Mothana *et al.*, 2005[2], Bajpai *et al.,* 2005[3]; Wojdylo *et al.,* 2007[4]. Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth (anti infectious arthritis activity) according to Chopra *et al.,* 1992[5]; Bruneton *et al.,* 1995[6]. Therefore, these plant drugs deserve detailed studies in the light of modern science.

Elephantopus scaber is a coarse, rigid, erect, hairy herb 30 to 60 cm high. Stems are forked and stiff. Leaves are mostly in basal rosette and oblong-ovate to oblong-lancelike, 10-25 cm in length and often very much notched on the margins decoction of *E.scaber* is widely used to treat diarrhea, dysentery, stomach troubles and blood vomiting in tuberculosis according to Bhattarai, 1989[7]; Taylor *et al.,* 1995[8] and root paste has been used externally as anti venom, antiseptic for cuts and wounds, lesions for chicken pox, antipyresis (2 teaspoonfuls of the root paste three times a day for 2-3 day) while its fresh roots are chewed to treat cough, cold and headache according to Bhattarai, 1989; Bhandary *et al.,* 1995[9]; Taylor *et al.,* 1995.

The aim of the present study is to investigate the antioxidant and anti microbial properties of *Elephantopus scaber.* In this study we are reporting the results in order to orient future investigations towards the finding of new potent and safe antioxidant and anti infectious compounds.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used were of analytical grade. Folin-Ciocalteau reagent, 1, 1- diphenyl-2-picrylhydrazyl were purchased from Sigma Chemical Company, St. Louis, USA), Ascorbic acid from Loba Chemie Pvt.ltd, Mumbai. Other chemicals Rifampicin, Griseofulvin, DMSO, Muller Hinton agar media were purchased from Sisco Research Laboratories Pvt Ltd., Mumbai.

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Test organisms

Five bacterial and three fungal species were used. The bacterial species were purchased from National collection of industrial micro organisms (NCIM), Pune. The Bacterial species were maintained in the nutrient broth medium on placing shaker in separate culture tubes for each species separately. Out of five, one is gram positive organisms ***(****Staphylococcus aureus)* and other four belonging to gram negative *(Escherichia coli, Klebsiella pneumonia, Pseudomonas aerogenosa, Salmonella typhimurium).* Fungal species include *Candida spp (C.bombii, C. tropicalis, C.utilis).*

Culture media

For Antiseptic arthritic activity of *Elephantopus scaber* Muller-Hinton Agar media (Solid and Broth) was used. For maintaining the bacterial species Nutrient broth was used.

Plant material

The plant material used in present study is collected from Puchikapadu, Jellugumelli mandal, West Godavari Dist, Andhra Pradesh, India and authenticated by the taxonomist Dr.Prayaga Murthy Pragada, Depart of Botany, Andhra University; a specimen was deposited in the herbarium of Andhra University.

Preparation of extracts

Freshly collected plant material was dried under shade and the dried material was milled to obtain a coarse powder. To the coarse powder (1kg) in round bottomed flask 1 litre of alcohol (70% v/v) was added and macerated for 24 hours at room temperature. The macerated powder was packed in a Soxhlet apparatus and subjected to continuous fractionation with 3 litre of alcohol (70% v/v).

The liquid fraction was collected and evaporated under reduced pressure by using rotary evaporator (Buchi R-210) until a soft mass obtained. The mass obtained was weighed in each case. The fraction was thoroughly air dried to remove all traces of the solvent.

The fractionation was carried out from crude hydro alcoholic fraction and hexane, ethyl acetate and methanol and fractions were collected.

Phytochemical analysis

Phytochemical studies were carried out for hydro alcoholic, hexane, ethyl acetate and methanol fractions of *Elephantopus scaber* to detect the presence of steroids, terpinoides, tannins, flavonoides, saponins, glycosides, amino acids etc following the described procedures according to Faraz *et al.,* 2003[10]; Harborne *et al.,* 1998[11]; Edeogo *et al.,* 2005[12].

Quantification of total phenols:

Total phenolic content was determined using the Folin-Ciocalteau reagent according to Singleton & Rossi, 1965**[13]**. Folin-Ciocalteau colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue absorption with a maximum at 765nm.The intensity of the light absorption at that wave length is proportional to the concentration of phenols. By using standard gallic acid calibration curve, the concentration of phenolic content was measured in gallic acid total equivalents using units mg/gm.

***In-Vitro* antioxidant activity**

All the fractions (Hydro-alcoholic, hexane, ethyl acetate and methanol) of *E.scaber* were screened for free radical scavenging activity against superoxide radical, hydroxyl and DPPH radicals at different concentrations. The percentage inhibition and 50% inhibition concentrations (IC_{50}) were calculated. All experiments were performed thrice and the results were averaged.

DPPH Radical

The DPPH radicals are widely used to investigate the scavenging activity of some natural compounds. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH by the reaction. The method helps to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm.

Resulting a color change from purple to yellow, the absorbance decreased when the DPPH was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule according to Mathaus 2002[14].

Superoxide Radical

Components are a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals according to Aurand and Boonme, 1977[15]. Furthermore, superoxide radical is considered to play an important role in the peroxidation of lipids according to Dahl and Richardson, 1978[16]. At low pH value superoxide will protonate to form the perhydroxyl radical ($HO_{2\cdot}$), a more reactive oxidizing species but at physiological pH less than 1%will be in protonated form according to Yun-zhong Fang *et al*., 2002[17].

Hydroxyl Radical

The reactive oxygen radicals are unstable and react readily with other groups or substances in the body, resulting in cell damage and hence human diseases according to Halliwell and Gutteridge, 1989[18]. Among the oxygen radicals specifically, the hydroxyl radical is the most reactive.

It severely damages adjacent biomolecules such as all proteins, DNA, PUFA, Nucleic acid, and almost any biological molecule it touches. This damage causes ageing, cancer and several diseases according to Aruoma, 1989[19]. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases.

Calculation of Percentage Inhibition

The percentage inhibition of superoxide production by the extract was calculated using the formula:

$$\text{Inhibitory ratio} = \frac{(A_o - A_1) \times 100}{A_0}$$

Where, A_0 is the absorbance of control; A_1 is the absorbance with addition of plant extract/ ascorbic acid.

Calculation of 50% Inhibition Concentration

The optical density obtained with each concentration of the extract/ascorbic acid was plotted taking concentration on X-axis and percentage inhibition on Y-axis. The graph was extrapolated to find the 50% inhibition concentration of extract/ ascorbic acid.

Statistical Analysis

Values were expressed as means ± standard deviation. Analysis of variance was conducted and differences between variables were tested for significance by one-way ANOVA and linear regression analysis was used to calculate IC_{50} values. All determinations were done at least in triplicate and all were averaged.

***In-Vitro* Anti microbial Activity**

In-vitro anti microbial activity is determined by cup-plate method. For this study, the author has precisely chosen microorganisms causing septic arthritis. In-vitro anti microbial study has been performed for the first time. The method involves inhibition of microbial growth by test extract using cup-plate method. In this method Muller Hinton Agar medium was prepared and autoclaved at 15 lbs pressure at 121^{0}C for 15 to 20 min, later the Muller-Hinton Agar medium was cooled at room temperature. Then 20 ml of Muller-Hinton Agar medium is taken in eight test tubes, to those tubes subjected to testing bacterial inoculums (20µl). After adding the inoculums the tubes were mixed well for equal distribution of the Bacterial species in the medium wells were prepared by using metal steel borer. Different concentrations of plant fractions were placed in the wells of solidified Petri dishes.

Then the plates were incubated in incubator for 24hrs at 36^{0}C. After incubation the zones of inhibitions were measured in mm according to Bauer, A.W *et al.,* 1966[20] for each treatment nine replicates were maintained.

The anti infectious arthritis activity is determined by agar-cup plate method by Patel & Patel (1956) Standard. Media was prepared or readymade media was also available like Muller Hinton agar media.

The cup plate assay of drug potency is based on measurement of the diameter of zone of inhibition of microbial growth surrounding cylinders (cups), containing various dilutions of plant fraction (test compound).

RESULTS AND DISCUSSION

Phytochemical analysis

Qualitative chemical tests indicated that the all fractions (Hydro-alcoholic, hexane, ethyl acetate and methanol) of *Elephantopus scaber* showed positive test for Alkaloids, Flavonoids, Tannins, quinones, carbohydrates and oils. But the extracts do not contain the amino acids.

Quantification of total phenols

The phenolic content in hydro-alcoholic fraction, hexane, ethyl acetate and methanolic fractions of *E.scaber* was found to be 4.49, 3.39, 8.76 and 3.34 mg/g respectively. Among the selected fractions ethyl acetate fraction of *E.scaber* showed high phenolic content. The results are given in table 1.

Table 1. Phenolic content present in different fractions of *Elephantopus scaber* (mg/g)

Name of the fraction	GAE(Gallic acid equivalent) µg/gm
E.scaber hydro alcoholic fraction	4.49
E.scaber ethyl acetate fraction	8.76
E.scaber methanolic fraction	3.34
E.scaber hexane fraction	3.39

***IN-VITRO* Antioxidant activity**

DPPH Radical Scavenging Activity

In this study the hydro alcoholic, methanolic, ethyl acetate and hexane fractions of *E.scaber* was found to possess concentration dependent inhibition of DPPH radical scavenging activity. The fractions hydro-alcoholic fraction, methanolic, ethyl acetate and hexane fractions of *E.scaber* showed concentration dependent percentage inhibition of DPPH radical and better percentage inhibition was produced at a concentration of 640 µg/0.1ml. The results are shown in fig 1.

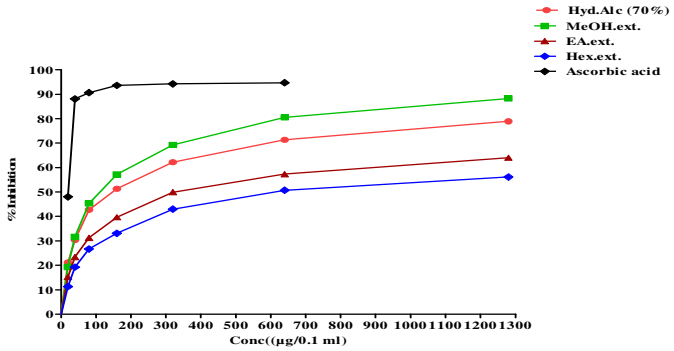

Fig 1. Concentration dependent percent inhibition of DPPH radical by different fractions of *Elephantopus scaber* and Ascorbic acid in *In-vitro* studies

Superoxide Radical Scavenging Activity

In this study the hydro alcoholic, methanolic, ethyl acetate and hexane fractions of *E.scaber* was found to possess concentration dependent inhibition of superoxide radical scavenging activity. The hydro-alcoholic, methanolic, ethyl acetate and hexane fractions of *E.scaber* showed concentration dependent percentage inhibition of superoxide radical and better percentage inhibition was produced at a concentration of 640 µg/0.1ml. The results are shown in fig 2.

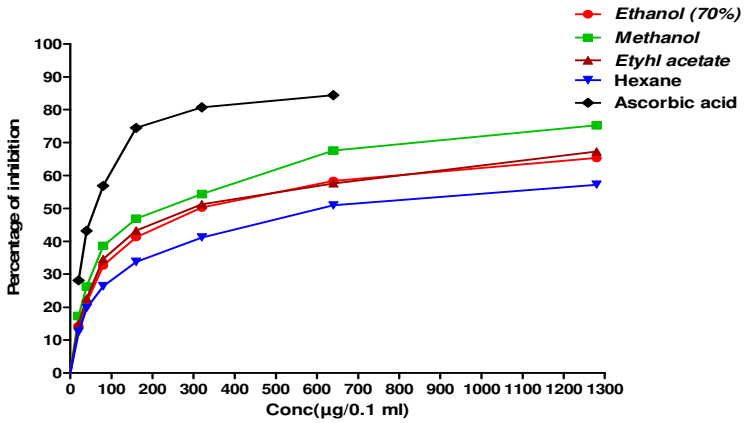

Fig 2. Concentration dependent percent inhibition of superoxide radical by different fractions of *Elephantopus scaber* and Ascorbic acid in *In-vitro* studies

Hydroxyl Radical Scavenging Activity

In this study the hydro alcoholic, methanolic, ethyl acetate and hexane fractions of *E.scaber* was found to possess concentration dependent inhibition of superoxide radical scavenging activity. The hydro-alcoholic , methanolic, ethyl acetate and hexane fractions of *E.scaber* showed concentration dependent percentage inhibition of superoxide radical and better percentage inhibition was produced at a concentration of 640 µg/0.1ml. The results are shown in fig 3.

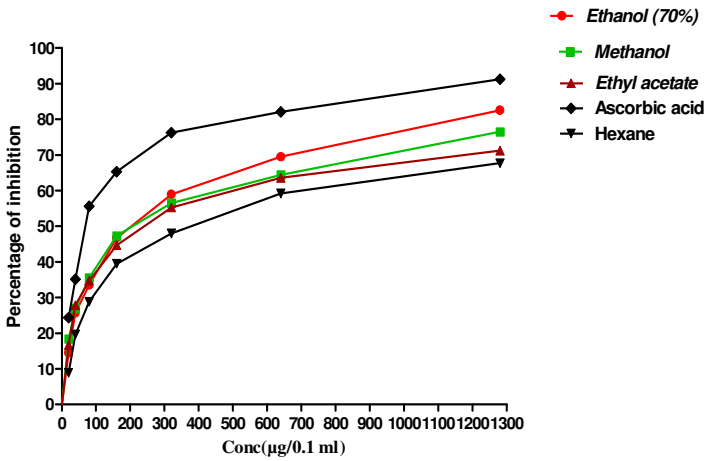

Fig 3. Concentration dependent percent inhibition of Hydroxyl radical by different fractions of *Elephantopus scaber* and Ascorbic acid in *In-vitro* studies

Ic_{50} VALUES

The Ic_{50} value for superoxide, DPPH, hydroxyl radical showed better activity for hydro-alcoholic fraction than hexane fraction, methanolic and ethyl acetate fractions of *E.scaber.* The results are given in table 5 and figure 4.

The order of DPPH radical scavenging activity is as follows:

Ascorbic acid (16µg) > methanolic fraction of *Elephantopus scaber* (112µg) Hydro alcoholic fraction of *Elephantopus scaber* (147µg) > ethyl acetate fraction of *Elephantopus scaber* (313µg.) > hexane fraction of *Elephantopus scaber* (602µg).

The order of superoxide radical scavenging activity is as follows:

Ascorbic acid (59.3µg) > methanolic fraction of *Elephantopus scaber* (212µg) > ethyl acetate fraction of *Elephantopus scaber* (280µg) > Hydro alcoholic fraction of *Elephantopus scaber* (314µg) > hexane fraction of *Elephantopus scaber* (596µg).

The order of hydroxyl radical scavenging activity is as follows:

Ascorbic acid (66µg) > methanolic fraction of *Elephantopus scaber* (194µg) > Hydro alcoholic fraction of *Elephantopus scaber* (197µg) > ethyl acetate fraction of *Elephantopus scaber* (245µg) > hexane fraction of *Elephantopus scaber* (392µg).

Table 5. *In-vitro* 50% inhibition concentration (IC_{50}) of different fractions of *Elephantopus scaber* on DPPH, Superoxide and Hydroxyl free radicals.

	DPPH radical	Superoxide radical	Hydroxyl radical
Hyd. Alc.ext.	147	314	197
MeOH.ext.	112	212	194
EA.ext.	313	280	245
Hex.ext.	602	596	392
Ascorbic acid	16	59.3	66

In –vitro Anti microbial Activity

All the fractions (hexane, ethyl acetate, methanol and hydro alcoholic fractions) showed highly significant activity at a concentration of 1mg/100µl. Of the entire fractions ethyl acetate fraction showed more activity towards *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* than the other test organisms *E.coli, S.aureus, S. typhi and Candida species.* Results are given in tables 6 to 10.

Table 6. Anti-bacterial activity of *E.scaber* hexane fraction

Concentrations (per 100 µl)	Zone of inhibition in mm				
	Ec	*Sa*	*Kp*	*Pa*	*St*
50µg	7	9	8	6	7
100µg	8	11	13	12	9
200µg	10	12	18	16	11
500µg	12	13	23	20	13
1mg	16	17	28	23	16
Rifampicin	18	22	35	26	24
DMSO(control)	0	0	0	0	0

Table 7. Anti-bacterial activity of *E.scaber* ethyl acetate fraction

Concentrations (per 100 µl)	Zone of inhibition in mm				
	Ec	*Sa*	*Kp*	*Pa*	*St*
50µg	8	8	8	5	6
100µg	9	10	12	11	8
200µg	11	11	17	14	10
500µg	13	12	21	19	12
1mg	17	14	26	22	15
Rifampicin	18	22	34	25	23
DMSO(control)	0	0	0	0	0

Note: *Ec-Escherichia coli, Sa-Staphylococcus aureus, Kp- Klebsiella pneumonia, Pa-Pseudomonas aerogenosa, St-Salmonella typhimurium*

Table 8. Anti-bacterial activity of *E.scaber M*ethanol fraction

Concentrations (per 100 µl)	Zone of inhibition in mm				
	Ec	*Sa*	*Kp*	*Pa*	*St*
50µg	6	7	6	7	6
100µg	7	8	10	8	7
200µg	9	10	14	11	8
500µg	11	11	17	16	10
1mg	15	16	21	20	13
Rifampicin	17	21	32	24	22
DMSO(control)	0	0	0	0	0

Table 9. Anti-bacterial activity of *E.scaber* Hydro alcoholic fraction

Concentrations (per 100 µl)	Zone of inhibition in mm				
	Ec	*Sa*	*Kp*	*Pa*	*St*
50µg	5	7	7	6	6
100µg	6	8	11	10	7
200µg	8	10	16	12	9
500µg	10	13	18	17	11
1mg	14	15	23	21	14
Rifampicin	17	20	35	24	22
DMSO(control)	0	0	0	0	0

Note:*Ec-Escherichia coli, Sa-Staphylococcus aureus, Kp- Klebsiella pneumonia, Pa-Pseudomonas aerogenosa, St-Salmonella typhimurium*

Table 10. Anti fungal activity of *Elephantopus scaber*

Concentration (Per µl)	Zone of inhibition in mm											
	Elephantopus scaber (hexane fraction)			*Elephantopus scaber* (ethyl acetate fraction)			*Elephantopus scaber* (methanol fraction)			*Elephantopus scaber* (hydro alcohol fraction)		
	Cb	*Ct*	*Cu*	*Cb*	*Ct*	*Cu*	*Cb*	*Ct*	*Cu*	*Cb*	*Ct*	*Cu*
50µg	-	-	-	-	-	-	-	-	-	-	-	-
100µg	-	-	-	-	6	7	-	6	6	-	-	-
200µg	-	-	-	7	9	14	6	8	8	7	6	7
500µg	8	6	6	13	15	19	10	12	12	12	10	11
1mg	15	13	12	17	18	21	14	17	18	16	13	14
Griseofulvin	19	20	22	18	19	22	19	21	21	19	21	22
DMSO(control)	0	0	0	0	0	0	0	0	0	0	0	0

Note: *Cb: Candida bombii; Ct: Candida tropicalis; Cu: Candida utilis*

CONCLUSION

In the present study, antioxidant activity of test extracts was determined and was found to possess concentration dependent inhibition using DPPH, superoxide and hydroxyl radicals scavenging activity. *In-Vitro* Anti microbial Activity of the same test extracts was observed using cup-plate method by measuring the diameter of zone of growth inhibition against arthritis causing microorganisms. Highly significant zone of inhibition was exhibited by the ethyl acetate and hexane fractions of *E.scaber* against all test organisms (bacteria and fungi). Total phenolic and alkaloid content was determined showing variation in the fractions. The process on the study of anti-inflammatory activity is still an on-going process.

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