

ORIGINAL ARTICLE

Antioxidant Potential and Validation of Bioactive B-Sitosterol in *Eulophia campestris* Wall.

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ABSTRACT

Since ancient time, Medicinal plants are using in many herbal provision. Increasing demand of medicinal plants will lead to adulteration, and thus there is a worldwide need to enlarge the quality of plant material. There has been increasing interest in the valuable health effects of overwhelming fruits and vegetables. Mainly, the presence of phenolic antioxidants is believed to have the defending mechanisms. In the present study the plant of *Eulophia campestris* Wall. was selected. The total phenolic content of the extracts was indomitable by antioxidant activity of the methanolic extracts and was assayed through some in vitro models such as antioxidant capacity by radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. In our preliminary screening, the methanol extract of the plant were subjected to antioxidant activity which shows moderate to potent antioxidant activity, with the ED₅₀ value i.e. 1.593 µg/ml. Further, high content of alkaloids possibly will account for the antioxidant activity of the plant. This Study shows the impending of the methanolic extract of *Eulophia campestris* Wall. as a natural antioxidant. Also, β-Sitosterol was isolated from the petroleum ether soluble fraction of the extract of *Eulophia campestris* Wall.

Keywords: β-Sitosterol, Antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Scavenging activity

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INTRODUCTION

Eulophia campestris Wall. was used to be made from the dried tubers of several species of Orchis and related genera such as the *Anacamptis pyramidalis*. The tubers are gathered, scalded, and dried quickly and this process removes their bitterness and disagreeable odour, as well as renders them somewhat translucent. The stem of the plant is 1 to 3 feet. At the apex of the stem there arise the 2 to 6 inch long ovate shaped leaves. The flower presentation is 1 to 6 inch long that has 2/3 inch long flowers which are of purple colour. The rhizome is round which is yellow to brown in colour. It is vata pitta suppressant and it strengthens the nervous system and also brain. It helps in gaining strength in the body and avoids the weakness. The parts that are most frequently used are the stems and the bulbs because they are said to be the life of the plants. Once they are dried up they can be powdered and used as desired. The powder of plant is being used mainly as the aphrodisiac agent and improves the general health condition of the body. The flower, on the other hand, is also dried and used as tea. This product is considered to be excellent and extremely exclusive. *Eulophia campestris* Wall have phytochemicals and antioxidant properties that can be used to cure various illnesses.

Highly reactive free radicals produced by exogenous chemicals otherwise endogenous metabolic processes in the human body are accomplished of oxidizing biomolecules that are significant in the mutagenic changes, cancer, cell death as well as tissue damage[1]. Antioxidants play an indispensable role in the anticipation of diseases and have capacity to diminish oxidative stress by chelating trace elements or scavenging free radicals along with protecting antioxidant defenses [2,3]. The antioxidative effect was largely due to the phenolic components, such as phenolic acids as well as phenolic diterpenes [4]. The antioxidant activity of phenolic compounds is generally due to their redox properties, which can also plays a significant role in absorbing as well as neutralizing free radicals, quenching singlet in addition to triplet oxygen, or decomposing peroxides⁵. The present study was planned to examine the antioxidant potential and validation of bioactive β-Sitosterol in *Eulophia campestris* Wall.

MATERIALS AND METHODS

Collection

Authentic samples: Various market samples of *Eulophia campestris* Wall. were procured from Chunnal Attar Ayurvedic Store, Ghat Gate, Jaipur in the month of March, 2010.

Identification:

All the samples were authenticated and were given identification number. The identification was as follows:

These samples were authenticated and submitted in Ethnomedicinal Herbarium, Centre of Excellence funded by DST, MGias, Jaipur (Rajasthan).

Processing of plant materials:

During the course of the study each sample was screened for its foreign matter and milled, before use.

Experimental details:

Present studies were performed on *Eulophia campestris* Wall. for the following studies-

1. Phytochemical test of plant extract
2. High pressure liquid chromatography (HPLC)
3. Antioxidant Potentials of Methanolic extract of plant

1. PHYTOCHEMICAL SCREENING

Phytochemical screening was performed using standard procedure:

Test For Reducing Sugars (Fehling's Test)

The aqueous ethanol extract (0.5gm in 5 ml of water) was added to boiling fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction.

Test for Terpenoides (Salkowski Test)

To 0.5 gm each of the extract was added to 2ml of chloroform. Concentrated sulphuric acid (3ml) was carefully added to form a layer. Reddish brown coloration of the interface indicates the presence of terpenoides.

Test for Flavonoides

4ml of extract solution was treated with 1.5ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated Hydrochloride acid was added and red colour was observed for flavonoids and orange color for flavons.

Test for Tannins

About 0.5 g of the extract was boiled in 10ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for Saponins

To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for Alkaloids

Alkaloids solutions produce white yellowish precipitate when a few drops of Mayer's reagents are added. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent.

The alcoholic extract was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of mayer's reagent. The sample was then observed for the turbidity or yellow precipitation.

2. High pressure liquid chromatography (HPLC)

In the present work various Indian medicinal plants were subjected onto the HPLC analysis using Shimadzu Model LC2010 A_{HT} Auto Sampler (UV-VIS Detector).

Columns

There are various columns such as guard, derivatizing, capillary, fast and preparatory columns. In the present work, Hypersil BDS C18 column were used. This column has a large column diameter which is designed to facilitate large volume of injection as compared to analytical ones. The back pressure regulator is placed immediately posterior to the HPLC detector which generates constant pressure to the detector outlet and prevents the formation of air bubbles within the system to protect the column from damage. Thus the baseline is enhanced. Packing for columns are diverse, since there are many modes of HPLC. They are available in different sizes, diameter and pore sizes. In the present studies, column size (250 × 4.6 mm) and 100 Å pore size, 5 µm particle diameters was used to performed various analysis.

Mobile Phase

The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected onto the

mobile phase of an assay through the injected port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample and thus, will have variable retention times. There are several types of mobile phases, these include: isocratic gradient, and polytypic. In the present study 15 V Ethanol : 85 V Acetonitrile were used as mobile phase to evaluate better resolution of chromatograms and their co-comparison.

Stationary Phase

The stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. Column containing various types of stationary phase are commercially available. Some of the more common stationary phase include liquid-liquid, liquid-solid (adsorption), size exclusion, normal phase, reverse phase, ion exchange and affinity.

The reverse phase operates on the basis of hydrophilicity and lithophilicity. The stationary phase consists of silica based packing with an alkyl chain covalently bound. For example C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus, hydrophilic compounds elute more quickly than do hydrophobic compounds.

Injectors for HPLC

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a micro-syringe (20 μ l) and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 μ l to over 500 μ l. In the present work 20 μ l loops were used to carry out the studies. Injection was given by a specific syringe with a blunt tip.

HPLC Pumps

There are several types of pumps available for use with HPLC analysis viz: reciprocating piston pumps, syringe type pumps and constant pressure pumps. Syringe type pump are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500 ml. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor and in the present study LC Solution (Shimadzu) liquid chromatography pumps were used.

Detectors and Detection limits

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and in most cases the detection and sensitivity parameters may also be controlled. There are many types of detectors that can be used with HPLC. Some of the more common detectors include: refractive index (RI), ultra-violet (UV), fluorescent, radiochemical, etc.

Ultra-violet (UV) detectors measure the ability of a sample to absorb light. This can be accomplished at several wavelengths: UV detectors have sensitivity to approximately 10^{-8} or 10^{-9} g/ml. It can have fixed wavelength measures at one wavelength, usually 254nm, variable wavelength measures at one wavelength at a time, but can detect over a wide range and/ or diode array measures a spectrum of wavelengths simultaneously. In the present work, the UV detector was used for chromatogram analysis of various species.

Flow Rate

The speed of solvent by which it moves in the column varies according to different modes where as in the present studies, attempts have been made to analyze the chromatograms with 1ml/min flow rate was used. The Wave length which was used is 254nm and the column temperature was ambient.

Sample preparation for HPLC chromatograms

HPLC is used in identification of antioxidant compounds present in the various medicinal plants. Dried powdered plants of selected species were extracted (6 hour) in petroleum ether, filtered and concentrated to dryness. For HPLC analysis, 1 mg extract of each drug were dissolved in 1 ml methanol

and used for fingerprinting analysis. 20 µl of each sample was subjected onto Shimadzu HPLC system in an analytical mode. Various peaks were observed at different retention time (rt) and each rt reflects a compound.

3. Antioxidant Activity

Preparation of test extracts

All the test plant sample and their adulterants were milled and refluxed in ethanol for 36 h, filtered, concentrated to dryness *in vacuo*. A portion of ethanolic extract was further successively extracted in petroleum ether, benzene, chloroform, alcohol and water, concentrated and stored at minimum temperature, until used.

Preparation of DPPH

DPPH (1, 1'-diphenyl-2-picrylhydrazyl, $C_{18}H_{12}N_5O_6$; Hi media) 0.8 mg was dissolved in 10 ml methanol to obtain a concentration of 0.08 mg/ml for antioxidative (qualitative and quantitative) assay.

Qualitative assay

Each successive extract (10 mg) was dissolved in 10 ml of its suitable solvent to get a concentration of 1 mg/ml and from this; 0.25 µl was taken with the help of micropipette, applied on silica gel G coated plates. These circular spots were sprayed with DPPH solution, allowed to stand for 30 min. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced, and the changes in colour (from deep- violet to light- yellow on white) were recorded at 517 nm on a UV spectrophotometer (Varian Cary PCB 150, Water Peltier System).

Quantitative assay

A concentration of 1 mg/ml of ethanolic extract of each test sample was prepared to obtain different concentrations ($10^2 \mu\text{g}$ to $10^{-3} \mu\text{g}/\text{ml}$). Each diluted solution (2.5 ml each) was mixed with DPPH (2.5ml). The samples were kept in the dark for 15 min at room temperature and then the decrease in absorption was measured. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured. The UV absorbance was recorded at 517 nm. The experiment was done in triplicate and the average absorption was noted for each concentration. Data were processed using EXCEL and concentration that cause 50% reduction in absorbance (RC_{50}) was calculated. The same procedure was also followed for the standards- quercetin and ascorbic acid.

RESULTS AND DISCUSSION

1. Phytochemical screening:

Table 1: Showing phytochemical screening results of *Eulophia campestris* Wall.

<i>Eulophia campestris</i>						
TEST	Reducing Sugar	Saponin	Tannin	Terpenoides	Flavonoides	Alkaloides
	-ve	-ve	-ve	-ve	-ve	+

The phytochemical screening of *Eulophia campestris* shows the occurrence of Alkaloids whereas it shows the absence of Reducing sugar, saponin, tannin, terpenoids and flavonoids respectively. The screening of the *Eulophia campestris* make only a small amount of differences in the constituent of the toughened plants. The drug shows the confirmation of strong antioxidant activity in more or in a less important amount. The existence of alkaloids in this plant is credible to be scrupulous for the free radical scavenging effects hardnosed.

2. High pressure liquid chromatography (HPLC):

In the present study, HPLC was run using methanol under 254 nm and was performed for various bioactives and thus β -Sitosterol was observed at rt time recorded at 18.138 which showed that as the column size increases it affects on retention time (column size \propto rt). It also affects the peak sharpness. In *Eulophia campestris* various peaks were observed at retention time i.e. 2.885, 3.193, 3.321, 3.493, 4.075, 18.138(β -Sitosterol).

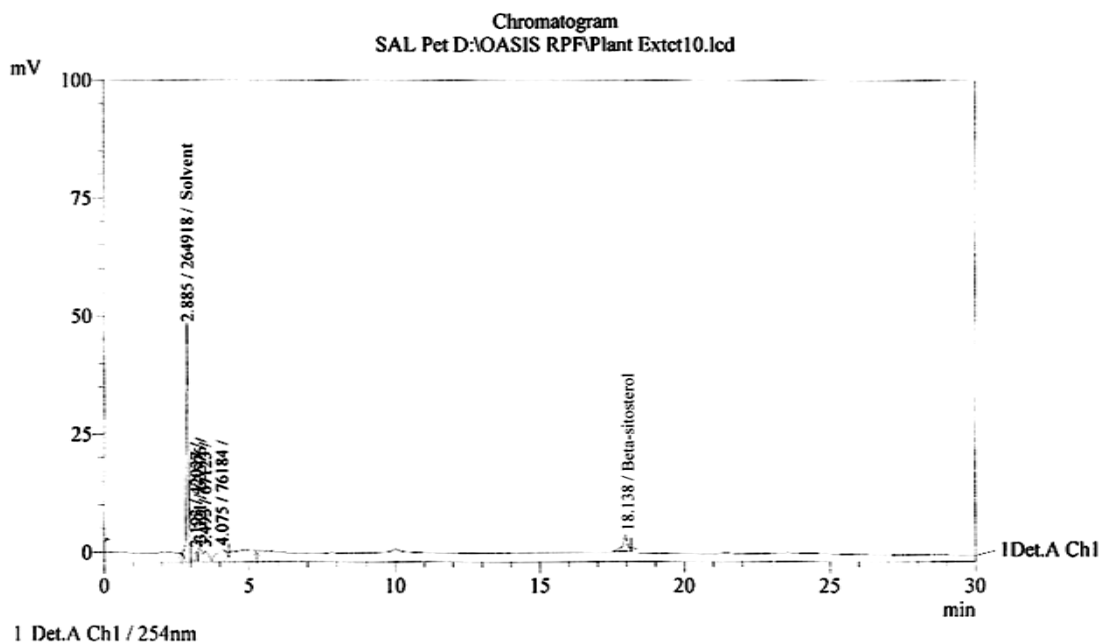


Fig 1: HPLC chromatograms of *Eulophia campestris* petroleum ether extract.

Table 2: Showing HPLC retention time and area of *Eulophia campestris* petroleum ether extract.

Peak#	Retention Time	Area	Height	Area%	Height%	K'
1.	2.885	264918	51767	48.015	68.993	0.000
2.	3.193	42037	6040	7.619	8.050	0.107
3.	3.321	63576	6584	11.523	8.775	0.151
4.	3.493	67123	4773	12.166	6.361	0.211
5.	4.075	76184	2537	13.808	3.381	0.413
6.	18.138	37900	3331	6.869	4.440	0.963

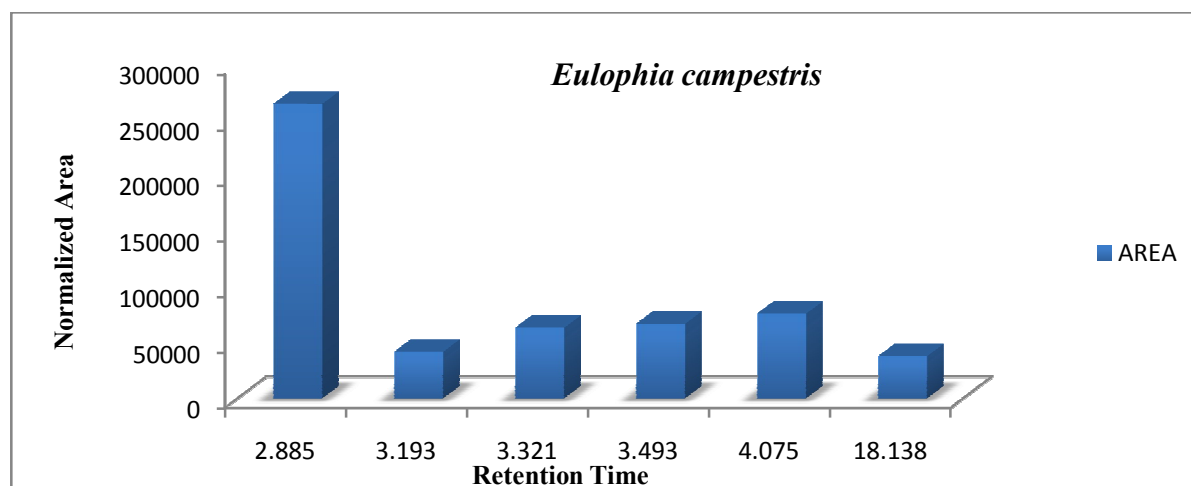


Fig 2: Normalized fingerprints of alcohol soluble *Eulophia campestris* extract.

3. Antioxidant Activity:

In the present investigation it was showed that the maximum optical density comes out to be 1.744 nm which is at the concentration $10^2 \mu\text{g/ml}$ and the smallest optical density is 0.969 nm which is at the

concentration $10^3 \mu\text{g/ml}$ where as the other shows comparable O.D at different concentrations i.e. 1.450 nm at $10^{-3} \mu\text{g/ml}$, 1.592 nm at $10^{-2} \mu\text{g/ml}$, 1.381 nm at $10^{-1} \mu\text{g/ml}$, 1.419 nm at $1 \mu\text{g/ml}$, 1.443 nm at $10^1 \mu\text{g/ml}$.

Table 3: Showing Optical density of *Eulophia campestris* on different concentrations

CONCENTRATION ($\mu\text{g/ml}$)	O.D (nm)
0.001	1.450
0.01	1.592
0.1	1.381
1	1.419
10	1.443
100	1.744
1000	0.969

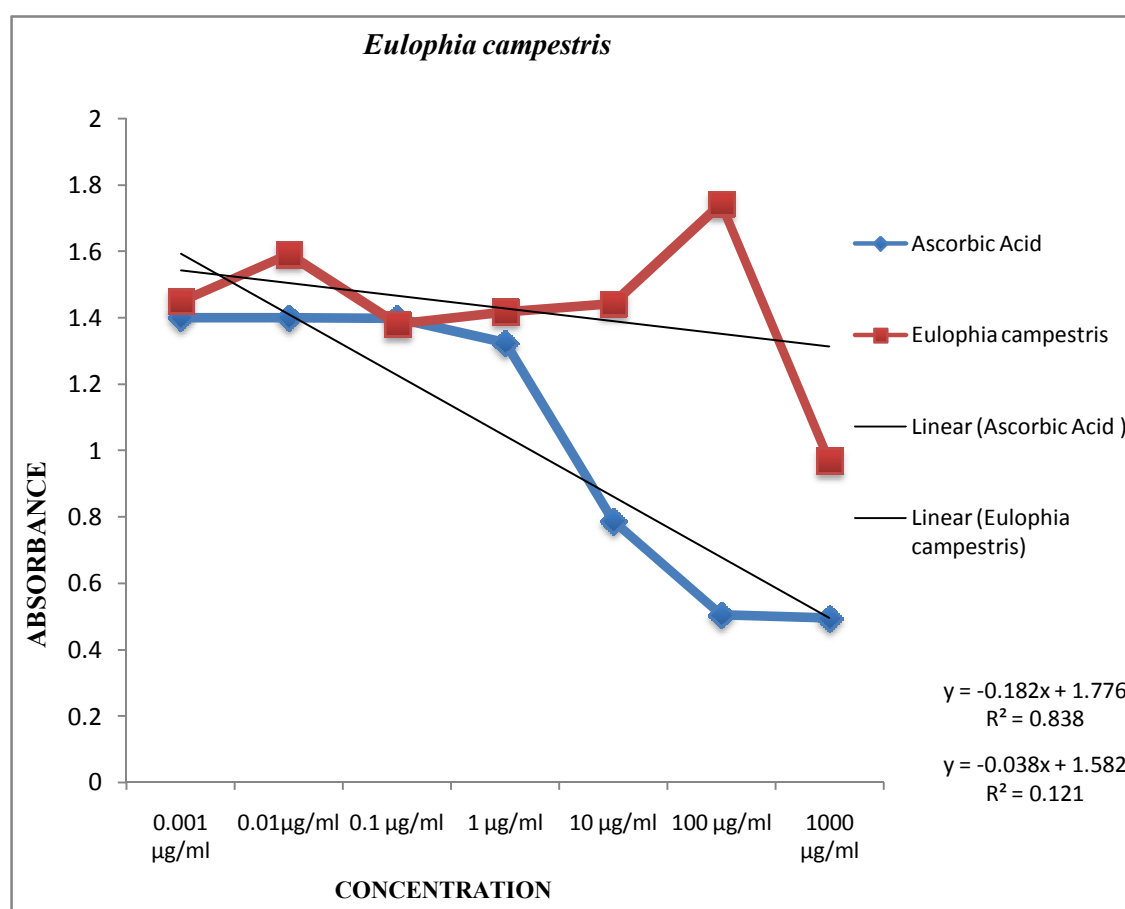


Fig 3: Graph showing Antioxidant Activity of *Eulophia campestris* at different concentration.

In the present investigations antioxidant activity of *Eulophia campestris* showed appreciable activity against the DPPH assay method where the regression line clear cut showed the effectiveness of it as it's have potentials which are comparable to ascorbic acid. The antioxidant activity of *Eulophia campestris* in methanolic extract using DPPH assay method⁶ shows appreciable activity comparable to standard ascorbic acid. The straight line showed $y = -0.182x + 1.776$ & regression = 0.838 whereas, in above drug the straight line is $y = -0.038x + 1.582$ & regression = 0.121.

CONCLUSION

For their plausible antioxidant activity, the extract of *Eulophia campestris* was subjected to screening. The consequential test systems, exclusively free radical scavenging next to with reducing power, was

used for the chemical analysis. Where HPLC is a rapid, reliable as well as data-oriented method which is used for quality control of a variety of drugs and provides enough characteristics that allow these to be distinguished. These peaks showed that there are different compounds and characteristic fingerprints for each drug to judge in herbal formulations. These normalized fingerprints are principal markers that can check the purity/impurity of drug at very low concentration. Further, the present study indicates that the alkaloids are present in *Eulophia campestris*. The occurrence of alkaloids in huge quantity is rationally proportional to the antioxidant activity so it is evidently show that occurrence of alkaloids will prove the antioxidant activity and promote a drug for treatment of various infectious disease. The occurrence of alkaloids in the plants is likely to be responsible for the free radical scavenging effects observed.

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