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STUDIES ON PHYTOCHEMICAL, ANTIOXIDANT, PROXIMATE AND ELEMENTAL ANALYSIS OF *HYBANTHUS ENNEASPERMUS* (L) F. MUELL LEAF.

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ABSTRACT

This study has investigated the phytochemical constituent, antioxidant properties, proximate and elemental analysis of different solvent leaf extracts (Hexane, ethyl acetate, ethanol, water) of H.enneaspermus known for its therapeutic value in folklore medicines. The Phytochemical and qualitative analysis on different extracts of H.enneaspermus have showed the presence of alkaloids, flavonoids, carbohydrates, steroids, tannins, glycosides and terpenoids. Ethanolic extract has demonstrated significant radical scavenging, antioxidant activity and highest phenolic content compared to hexane, ethyl acetate, and aqueous extracts. The dried leaf powder proximate analysis has revealed the presence of 90.6% of dry matter, 9.31% of moisture content, 35.1% of crude fiber, 12.5% of total ash, 0.3% of acid insoluble ash and 6.8% crude fat. Elemental analysis was done by ICP-OES and it revealed the presence of calcium (4.7mg), magnesium (4.2mg), zinc (3.0mg), phosphorus (3.2mg) and potassium (11.4mg) per gram of dry leaf powder.

KEYWORDS: Hybanthus enneaspermus, DPPH, Proximate analysis, ICP-OES.

INTRODUCTION

Man and his domesticated animals have been largely dependent on plants for the essentials of their existence by way of food, clothing, shelter and medicines etc, since the time immemorial. The demand for Ayurvedic /herbal drugs/ phytomedicines is increasing day by day globally**.** Traditional knowledge of medicinal plants has always been guided the search for new cures. In spite of the advent of modern high throughput drug discovery and screening

techniques, traditional knowledge systems have given clues to the discovery of valuable drugs. [1]

India has a rich heritage of medicinal plants contain a large amount of biologically active phenolic substances. [2-4] The formation of free radicals involved in the pathology of various diseases including diabetes, heart attacks, inflammation, neuro-degenerative diseases, cancers, obesity and ageing. [5] Antioxidants are effective in preventing free radical formation by scavenging them or promoting their decomposition and suppressing such disorders. The most practical way to fight degenerative diseases is to increase antioxidant activity in our body and that could be achieved by consumption of fruits, vegetables and edible plants. [6] There is an increasing interest in natural antioxidants present in medicinal and dietary plants which might help in the prevention of oxidative damage. Biologically active compounds like alkaloids, phenolic compounds, saponins, flavonoids, and many others, with known antioxidant properties, can be of great significance in therapeutic treatments. Therefore, researchers focused their interest towards herbal medicines in the treatment of diseases because of their minimal side effects and abundant availability.

Hybanthus enneaspermus, a traditional medicinal herb belonging to the family violaceae is distributed in the tropical and subtropical regions of the world. In India it is found in the warmer parts, from Uttar Pradesh southward to the Deccan Peninsula. In Ayurveda, it is known as ‘Sthalakamala’. It has common names like spade flower and pink lady’s slipper and popularly known as Ratanpurus (Hindi). The plant is cultivated particularly as a medicinal plant has been used in treatments and preventions of diverse diseases as folklore medicines. Traditionally the plant is used as anaphrodisiac, demulcent, tonic, diuretic, in urinary infections, diarrhea, cholera, leucorrhoea, gonorrhea, dysuria, inflammation and sterility. [7] The root sandals are employed for the bowel complaints of children. The leaves and tender stalks are demulcent and used as a decoction or electuary. They are employed in preparing a cooling liniment for head ache. An infusion of the plant is given in the case of cholera and decoction or powder of the whole plant is taken to improve memory, vitality and as a remedy for tuberculosis, asthma, fever and leprosy. Its infusion is good for all diseases of eyes. Fruit is used to treat scorpion sting. The plant has also been reported to have anti-inflammatory, antiplasmodial, antimicrobial, and anticonvulsant activity. [8-11]

Thus, in this present study the phytochemical constituents, antioxidant activity, proximate and elemental analysis had been investigated to prove the medicinal potentials of *H.enneaspermus.*

MATERIALS AND METHODS

Plant Collection: The *H.enneaspermus* was identified and authenticated by plant Taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh and voucher specimen no SVUBH/ 1060. The fresh leaves of *H.enneaspermus* were collected from Sesaschalam hills (Tirumala Hills) Chittoor district of Andhra Pradesh. Fresh leaves of H.enneaspermus were shade dried and milled to fine powder using a mechanical grinder.

Solvent Extraction: Organic solvents such hexane, ethyl acetate, ethanol and water were used for extraction. A sample, 100 g, was dissolved in 500 mL of respective solvents, incubated for 24 hrs and separated using separating funnel. The extract was then filtered with filter paper (Whatman No. 1) under reduced pressure using rota evaporator at 40°C. The concentrate was obtained to a dark molten mass then layered on aluminum foil and freeze dried for further use.

Extraction yields were weighted and calculated using the formula as followed:

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant powder}} \times 100$$

Phytochemical screening: Phytochemical examinations were carried out to detect the secondary metabolites (Alkaloids, saponins, carbohydrates, flavonoids, cardic glycosides etc.) in *H.enneaspermus* extracts by using standard procedures/methods as described by Trease and Evans. [4,12]

DPPH (2, 2-diphenyl-1-picrayl hydrazyl) free radical scavenging assay: Evaluation of antioxidant activity was done by using 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) method of Burits and Bucar. [13] Antioxidant reacts with DPPH and convert it to α,α-diphenyl,-β-picryl hydrazine. One ml of plant extract was added to 4ml of methanolic solution of 4mg of DPPH dissolved in 100ml of methanol. After 20-30’ incubation period at room temperature both DPPH solution with 2 ml of methanol used as sample (blank) and test samples incubated with different extracts absorbance was read against blank at 517nm. Inhibition of free radical by

DPPH in percent (1%) was calculated by using the following equation. The degree of decoloration indicates the scavenging potentials of the antioxidant extract.

%DPPH radical/scavenging = [(Δ inAbsorbance of control-test sample) / (Absorbance of control)] x100

Total antioxidant activity by Phosphomolybdenum Method: The total antioxidant capacity *H.enneaspermus* of different solvent extracts was evaluated according to the method of Prieto *et al.* [14] The absorbance of the samples were measured at 695 nm in UV spectrophotometer. The higher absorbance value indicates higher antioxidant activity. Ascorbic acid was used as standard for comparison.

Determination of Total phenolic content: The total phenolic content of plant extract was determined by using the Folin-Ciocalteu assay described by Singleton. [15] Gallic acid and double distilled water were used as standard and blank. The reaction mixture was incubated for 90 mins at room temperature, the absorbance against prepared reagent blank was determined at 750nm with in UV-Vis Spectrophotometer. The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/100g fresh weight. All samples were analyzed in triplicates.

Proximate analysis: The dried *H.enneaspermus* leaf powder was prepared for Proximate analysis. It includes preparation of the Dry matter, total ash, Crude fat, Crude fiber, Moisture content and Acid insoluble ash. The analysis was carried out using the AOAC methods. [16]

Quantitative Analysis of Elements in the *H. enneaspermus* leaf powder: Briefly, 0.5g of leaf powder was took in a silica crucible and heated in a muffle furnace till there is no evolution of smoke. The crucible was taken out, cooled at room temperature by keeping it in a desiccators and the ash was moistened with 10% nitric acid. The sample was made upto the mark volume in a volumetric flask with 2% nitric acid. Elemental analysis was done by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

RESULTS

Extraction Yield: Different solvents have different resolving strength towards the plant constituents which resulted in different yields as shown in (Table-1). The extraction of ethanol resulted with the highest amount of yield. *H.enneaspermus* leaves (100 g) used in ethanol solvent extraction obtained the highest yield (7.246%).

Table-1: Yield of *H.enneaspermus* leaves extracted using different types of solvents

Solvents	Weight of leaves used (g)	Yield (g)	Yield (%)
Hexane	100	1.432	1.432
Ethyl acetate	100	2.154	2.154
Water	100	3.765	3.765
Ethanol	100	7.246	7.246

Phytochemical analysis: Preliminary phytochemical screening of the leaf extracts of *H.enneaspermus* showed positive results for the presence of secondary metabolites like phytosterols, saponins, triterpenoids, alkaloids, carbohydrates, flavanoids, polyphenols and tannins. Bioactive compounds like phytosterols, alkaloids, carbohydrates and polyphenols were present in high amounts in ethanol extract (Table-2).

Table-2: Phytochemical screening of different extracts of *H.enneaspermus* leaf

S.No	Secondary Metabolites	Hexane Extract	Ethyl acetate Extract	Ethanol Extract	Aqueous Extract
1.	Phytosterols	+	+	+	+
2.	Triterpenes	+	+	+	+
3.	Saponins	-	-	+	-
4.	Alkaloids	+	+	+	+
5.	Carbohydrates	+	+	+	+
6.	Flavonoids	-	-	+	+
7.	Tannins	+	-	+	-
8.	Polyphenols	-	+	+	+

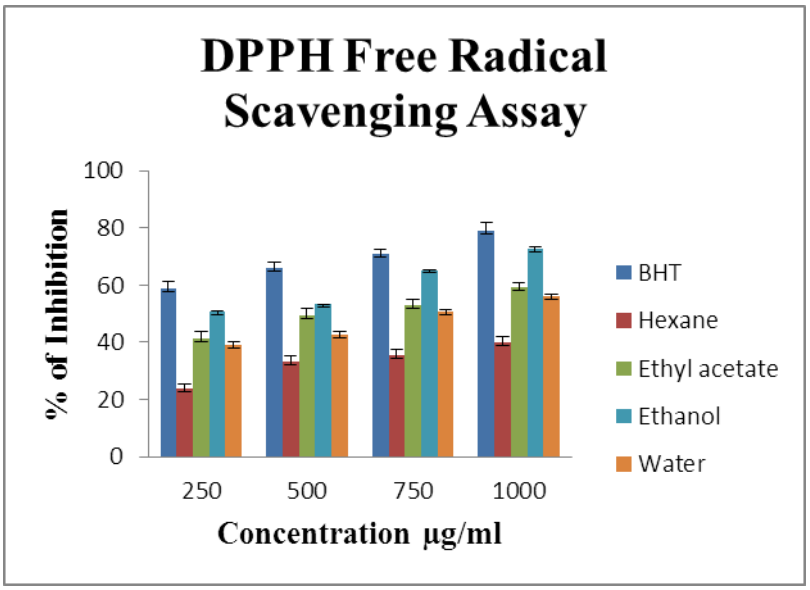
Note: "+" = presence and "-" = absence

Quantitative Analysis of Elements in the *H. enneaspermus* leaf powder: Elemental analysis was done by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The contents of Ca, Mg, K, Zn and P were analysed in leaf powder of H.enneaspermus. The concentration was expressed in mg/g of leaf dry powder (Table-3). Zinc 3, phosphorus 3.2, magnesium 4.2, calcium 4.7 and potassium 11.4mgs were found in gm of leaf powder.

Table-3: Quantitative Analysis of Elements in the *H. enneaspermus* leaf powder

S. No	Elements	Quantity(mg/g)
1.	Calcium	4.7mg
2.	Phosphorus	3.2mg
3.	Potassium	11.4mg
4.	Magnesium	4.2mg
5.	Zinc	3.0mg

DPPH (2, 2-diphenyl-1-picrayl hydrazyl) free radical scavenging assay: The hexane, ethyl acetate, ethanol and water extracts of *H.enneaspermus* leaves were analyzed for antioxidant property by using DPPH as recipient of radical. With increase in the range of 250 1000µg/ml of extract, the antioxidant activity was found to be increased in all extracts of hexane, ethyl acetate, ethanol, and water (Fig-1). The ethanolic extract showed the relatively highest actively compared to other preparations. BHT was used as standard.



Data are expressed as the mean of triplicate ± SD

Fig-1: Free radical (DPPH) scavenging activity of the *H.enneaspermus* leaf extracts

Total phenolic content: Phenols are very important plant constituents because of their scavenging ability of radicals due to their keto groups and it involves directly to antioxidative action. [17] In our present investigation it was found that ethanol extract had highest phenolic content followed by aqueous extract and hexane extract showed least quantity of phenols (Table-4). Total phenolic content was measured in conc. of total phenolics mg/ gAE/g of extract.

Phosphomolybdenum assay: The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherol, and carotenoids. Our results among hexane, ethyl acetate, ethanol and water, the ethanol shows the best antioxidant activity and the hexane shows the lowest activity (Table-4). Gallic acid was used as standard in this experiment.

Table-4: Total Phenolic content and Phosphomolybdenum activity of the *H.enneaspermus* leaf

Solvents	Total Phenolic content (mg/gAEs/g of extract)	Phosphomolybdenum assay (mg AE/g of plant extract)
Hexane	20.79±0.90	36.56±1.67
Ethyl acetate	28.47±0.97	43.49±1.14
Ethanol	41.17±0.75	60.02±1.97
Water	26.40±0.50	47.92±1.27

Proximate analysis: From our proximate analysis of plant sample includes that the total ash was 12.5% in dried leaf, the fat content showed was 6.8% and fibre content was 35.1% in plant samples (Table-5). In this study the analysis provided an insight into the composition of the tested *H.enneaspermus* in addition to its therapeutic potentials.

Table-5: Proximate analysis of *H.enneaspermus* leaf powder

S. No	Proximate factors (Parameters)	Dried *H.enneaspermus* leaf powder (% w/w)
1.	Dry Matter	90.6%
2.	Total Ash	12.5%
3.	Moisture content	9.31%
4.	Crude Fibre	35.1%
5.	Crude Fat	6.8%
6.	Acid insoluble ash	0.3%

DISCUSSION

Extraction of *H.enneaspermus* leaf was done in order to separate the biologically active portions of plant using selective solvents in to the solvents used. During extraction, solutes are diffused from solid plant material into the solvent used with similar polarity. As shown in Table 1, the ethanol extracts obtained the most yields. Therefore, the choice of solvent plays an important role to obtain the highest extracts yield. Phytochemical study has revealed that all tested plant bioactive constituents were present in the *H.enneaspermus* leaves ethanolic extract. The secondary metabolite constituents of *H.enneaspermus* detected include the alkaloids, flavonoids, saponins and tannins etc (Table-2). It is known that different phytochemicals have a broad range of pharmacological activities. For instance,pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects. [18] Steroids were used as allergy, arthritis and coronary failure therapy, control in menstrual cycle and increasing women fertility and tannins are reported to possess anti-irritant, anti-secretolytic, anti-phlogistic, antimicrobial and anti-parasitic effects. [19] Flavonoids and tannins are phenolic compounds and plant

phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. [20]

Results from antioxidant assays showed that the plant extracts possess antioxidant activity. The DPPH test provides information on the reactivity of the test compounds with a stable free radical. DPPH gives a strong absorption band at 517nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract. The ethanolic extract of *H.enneaspermus* appeared to be as potent as BHT which is used as standard. Plant phenolics are major group of compounds acting as primary antioxidants or free radical scavengers[21]. The ethanolic extract of *H.enneaspermus* showed higher phenolic than aqueous extract. Phenolic compounds normally contribute to quality and nutritional value in terms of modifying colour, taste, aroma, and flavor also in providing health beneficial effects. They also serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores[22]. Furthermore, proximate and elemental analysis showed that *H.enneaspermus* could serve as a good source of minerals, and fiber.

CONCLUSION

This present study has revealed the antioxidant activity, phytochemicals, proximate and elemental composition of *H.enneaspermus*. The presence of the identified phytochemicals makes the leaves pharmacologically active. Their antioxidant activity may be responsible for their usefulness in the management and treatment of various diseases. Proximate and elemental analysis has revealed that leaf nutrients are useful for many pharmacological activity. Further studies are to be carried out to isolate, characterize and elucidate the structure of the bioactive compounds from *H.enneaspermus* for the industrial drug formulations.

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