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Research Article

IN VITRO ANALYSIS OF ANTI-OXIDANT ACTIVITY AND ESTIMATION OF BIOMOLECULES IN THE LEAF EXTRACT OF *HYBANTHUS ENNEASPERMUS* (L).F. MUELL

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A B S T R A C T

Plant kingdom which provides by the basic necessity of food, clothes and shelter to the human beings. The use medicinal plants for curing diseases are one of the traditional practices in India which later become popular throughout the world. The plants in which bioactive compounds are present in high concentration are known as medicinal plants. The present study aims to analysis the antioxidant properties and estimation of primary and secondary metabolites in the leaf extract of *Hybanthus enneaspermus.*

INTRODUCTION

Medicinal plants are widely present in the world which is used to prevent and cure many diseases. Nearly 80% of world population depend medicinal plants for therapeutic purpose (Ngari *et al*., 2010; Wadood *et al*., 2013). Besides its therapeutic importance, medicinal plants also provide wide range of different chemical substances which can be developing as drugs. It is necessary to screen the bioactive compounds present in medicinal plants and also their biological activity, in order to reach the continuously demand of organic drugs (Sumathi and Parvathi, 2010).

Phytochemicals are bioactive compounds found in plants that work with nutrients and dietary fibre to protect against diseases. They are non-nutritive compounds (secondary metabolites) that contribute to flavor and colour (Johns, 1996). Many phytochemicals have antioxidant activity and reduce the risk of many diseases, for example, alkyl sulfide (found in onions and garlic), carotenoids (from carrots), and flavonoids (present in fruits and vegetables). Reactive oxygen-free radicals (ROS) have been implicated in many diseases and in aging process. These free radicals, which cause tissue damage via oxidative stress, are generated by aerobic respiration, inflammation, and lipid peroxidation.

Hybanthus enneaspermus (*L*.), a traditional medicinal her bbelonging to the family Violacea is distributed in the tropical and subtropical regions and well known for its therapeutic value (Thamizh Mozhi *et al*., 2013; Afolabi *et al*., 2014). The *H. enneaspermus* is considered to have great medicinal value and widely used to treat various diseases like Diabetics (Sudarsanam and Sivaprasad, 1995), Malaria (Antiplasmodial activity), Jaundice (Prakash *et al*., 1999), Male sterility (Aphrodisiac activity) (Priya *et al*., 2011; Patel., 2011)have reported that the plant is also used for urinary tract infections and the water retentionand plantsare used as tonic. In the present study, various solvent extracts of *H. enneaspermus* leaf was analyzed for phytochemicals using standard protocols.

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MATERIALS AND METHODS

Collection of plant sample*: Hybanthus enneaspermus* was freshly collected at early morning from Guru Nanak College, Velachery, Chennai, Tamil Nadu during the months of January (Fig. 1).

Figure 1 The whole plant of *Hybanthus enneaspermus*

The collected plant sample was washed and shade dried. The leaf was separated manually and powder by using mixer grinder. The fine leaf powder was preserved in the clean glass jar for further analysis.

Preparation of extracts using different solvents: Five grams of fine powder of *Hybanthus enneaspermus* (L.) leaf was extracted with ethanol, aqueous, acetone, chloroform and petroleum ether separately in order to extract the polar and non-polar compounds. The solution was transferred into boiling test tube and tightly closed with cotton wool. These solutions were kept on rotary shaker at 200-220 rpm for 24 hours. The supernatant was collected and solvent was evaporated to make the final volume and stored at 4^0C in air tight containers (Parekh and Chanda, 2007).

Qualitative analysis of antioxidant activity by DPPH method

The antioxidant activity of leaf extracts of *hybanthus enneaspermus* was determined by standard method (Hsiao, *et al*., 1996; Abirami and Muthuswamy, 2013).50 µl of leaf extracts of *Hybanthus enneaspermus* were taken in the microtiter plate separately. 100 µl of methanolic and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was added over the samples and incubated for 30 minutes in dark condition. BHT served as standard while methanol was taken as negative control. The samples were then observed for discoloration; from purple to yellow were considered to be strong and weak positive respectively. The samples were subjected for further quantitative analysis.

Quantitative analysis of free radical scavenging activity of Hybanthus enneaspermus leaf

100 µl of leaf extracts were mixed with 2.7 ml of methanol and then 100 µl of methanolic DPPH was added. The blend was incubated for 30 minutes in dark condition. Same amount of methanol served as blank. Subsequently, at every 5 minutes interval, the absorption maxima of the solution were measured using a UV double beam spectra scan (Chemito, India) at 517 nm. The antioxidant activity of the sample was compared with synthetic standard of (0.16%) of butylated hydroxy toluene (BHT).The capacity of scavenging free radicals was calculated as scavenging activity percentage (Sannigrahi, *et al.,* 2009; Gupta *et al*., 2012).

Calculation

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

Quantitative analysis of primary metabolites of Hybanthus enneaspermus leaf

Estimation of total carbohydrates:

The total carbohydrate was estimated by anthrone method Hedge and Hofreiter (1962). Hundred mg of the dried leaf powder was weighed and taken into a boiling tube. The solution was hydrolyzed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled in room temperature. This solution was neutralized with solid Na_2CO_3 until the effervescence ceases and made to 100 ml and centrifuged. The supernatant was collected and 0.5, 1ml aliquots were taken for analysis. Standard solution was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard '0' served as blank. The volume is made to 1 ml in all the tubes including the sample tubes by adding distilled water. Then 4 ml of anthrone reagent was added and this was heated for eight minutes in a boiling water bath and made to cool. Green to dark green color was recorded at 630 nm. A standard graph was drawn by plotting concentration of the standard versus absorbance. The amount of carbohydrate present in the tube was calculated from the graph. The Calculation is done by using this formula.

Calculation

$$\text{Carbohydrate in (100mg) sample} = \frac{\text{mg of glucose}}{\text{Vol. of test sample}} \text{ x } 100$$

Estimation of total proteins

Protein content of leaves was estimated by Lowry's method (Lowry *et al*., 1951). A series of standard solutions 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standards were pipette into test tubes 0.1 ml and 0.2 ml of the leaf extract was pipette in other test tubes. The volume was made to 1 ml by adding water in all the test tubes. The test tube with 1 ml of water served as the blank. 5 ml of reagentwas added to each tube including the blank. The blend was mixed well and allowed to stand for 10 minutes. Then 0.5 mlof reagent D was added, mixed and incubated at room temperature in the dark for 30 min. Blue color was developed. The readings were recorded at 660 nm. Standard graph was drawn and the amount of protein in the sample was calculated. Protein percentage was calculated in relation to fresh weight and dry weight basis.

Calculation

The amount of protein mg/g or 100 g sample was expressed.

Estimation of total lipids

The total lipid present in the leaf extract of *Hybanthus enneaspermus* was estimated by standard method (Jayaram, 1981). Hundred mg of leaf powder was mixed with 10 ml distilled water and the blend was transferred into a conical flask containing 30 ml of chloroform and methanol (2:1). The mixture was thoroughly mixed and left overnight at room temperature in dark for complete extraction. Later, 20 ml of chloroform mixed with 2 ml of water were added and centrifuged. Two layers separated, the lower layer of chloroform, which contained all the lipids, was carefully collected in the pre weighed glass voiles and the colored aqueous layer of methanol which contained all the water soluble substances and thick pasty outer face layer were discarded in each test sample. The chloroform layers were evaporated to dryness and weighed. The test was replicated thrice and their mean values calculated.

Quantification of secondary metabolites of Hybanthus enneaspermus leaf

Estimation of total phenols

Total phenolic content was estimated by Folin Ciocalteu's method. 1 ml of test sample and standard gallic acid (10, 20, 40, 60, 80, 100 µg/ml) was taken in a test tubes separately. 5ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent was mixed in the test tubes and shaken. After 5 minutes, 1.5 ml of 20 % Na_2CO_3 was added and volume made up to 10 ml with distilled water. It was allowed to incubate for 2 hours at room temperature. Intense blue color was developed and absorbance was measured at 750 nm. The blank was performed using reagent blank with solvent. Gallic acid was

used as standard. The calibration curve was plotted using standard gallic acid. The total phenolic content was expressed as mg of gallic acid equivalent weight (GAE) per 100 g of sample (Bhalodia *et al*., 2011; Patel *et al*., 2010).

Estimation of total tannins

One ml of leaf extract was mixed with 0.5 ml of folin-ciocalteau's reagent followed by addition of 1ml of Na_2Co_3 solution and 8 ml of distilled water. The mixture was allowed to stand for 30 minutes at room temperature. The supernatant was collected and absorbance was recorded at 725 nm using UV- Visible spectrometer. Increasing concentrations of tannic acid was prepared and the absorbance of tannic acid was plotted for a standard graph. The tannic acid content was expressed as mg tannic acid equivalent per 100 gm of sample (Hagerman *et al*., 2000).

Estimation of total flavonoids

Ten mg of quercetin was dissolved in 80% ethanol and then diluted to 25, 50, 75 and 100 mg/ml. 0.5 ml of test sample, 1.5 ml of methanol, 0.1 ml of aluminium chloride, 0.1 ml of potassium acetate and 2.8ml of distilled water were added and mixed well. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. Sample blank was prepared by replacing the amount aluminium chloride by distilled water. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract (Pal *et al*.,2009; Kiranmani *et al*., 2012; Naskar *et al*., 2011).

Estimation of total steroids

The standard steroid solution was prepared and added to 1ml of the leaf extract. The solution was transferred into 10 ml volumetric flask. 2ml of Sulphuric acid (4N) and 2 ml of Iron (III) chloride (0.5% w/v), was added followed by, 0.5 ml of Potassium hexacyanoferrate (III) solution (0.5% w/v). The mixture was heated in a water-bath and was maintained at 70±20°C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank (Narendra *et al*.,2013).

Estimation of total alkaloids

Total alkaloid content in *H. enneaspermus* leaf was determined by About 1 gm of sample was taken into 250 ml beaker, 100 ml of 10% acetic acid was added and covered it with aluminum foil. The mixture was allowed to stand for 4 hours. Content in beaker was filtered and the extract was concentrated on a water bath to a one quarter of the original volume. Conc. ammonium hydroxide was added dropwise to the extract up to the precipitation was complete. This solution was allowed to settle. The precipitated was collected, washed with dilute ammonium hydroxide and filtered by using Whatman filter paper. The remaining residue was alkaloid, completely dried in the oven at 40^0 C and finally weighed (Harbone, 1998).

Calculation

$$\text{Alkaloid (\%)} = \frac{\text{Final Weight of the sample}}{\text{Initial weight of the extract}} \times 100$$

Estimation of total terpenoids

One gm of sample was take in a test tube and soaked in alcohol for 24 hours. It was filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids (Ferguson,1956)

Estimation of total saponins

About 10 gm of *Hybanthus enneaspermus* leaves powdered was taken in a conical flask and then 50 ml of 20% ethanol was added. The solution was heated at 55^0c over a hot water bath for 4 hours. This mixture was filtered and the remaining residue re-extracted with 100 ml 20% ethanol. Both the extracts were combined and reduced up to 40 ml over water bath at 90^0c. The concentrate obtained solution was transferred into a 250 ml separating funnel, 10 ml of diethyl ether was added and shaken vigorously. In separating funnel, aqueous layer was recovered and the ether layer was discarded. To the aqueous extract 30 ml of n- butanol was added. A combinedn- butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample obtained were dried in oven to the constant weight and the percentage of saponins was calculated (Obadoni and Ochuko, 2001).

RESULTS AND DISCUSSION

Plants play important roles in discovery associated with new beneficial therapeutic agents and have received significant focus because of their bio- active substances like antioxidants, hypoglycemic and hypolipidemic factors. India has a prosperous record associated with applying different potent natural herbs and plant based components regarding management of different diseases. Herbal medicine is the use of plant extracts to treat various types of diseases.

Oxidative damages are either removed or repaired by host antioxidant defense mechanisms (Sun *et al.,* 1998) carried out by specific enzymes. Antioxidant properties are one of the most import claims for food ingredients, dietary supplements, cosmetics and anticancer natural products (Cherdshewasarta and Sutjit, 2008) since the therapeutic effects of several medicinal plant are usually attributed to their antioxidant phytoconstituents. It has been clearly stated that, an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases were determined (Yildrim*et al.,* 2001). The DPPH test provides information on the reactivity of compounds with a stable free radical DPPH that gives a

Table 1: Qualitative analysis of Anti-Oxidant activity in *Hybanthus enneaspermus* leaf extract with different solvents

Samples	Response
Co = Control	-
S = Standard (Butylated hydroxyl toluene)	++
Aq = Aqueous extract	+
E = Ethanol extract	++
A = Acetone extract	-
C = Chloroform extract	+
P.E = Petroleum Ether extract	_

Response: ++ = Strongly positive, + = Positive, - = negative

strong absorption band at 517 nm 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 decolourized as the colour changes from deep violet to light yellow (Brand-Williams, 1995).

Table 1, Fig. 2 shows the antioxidant property of *H. enneaspermus* leaf by changing color from purple to yellow of DPPH.

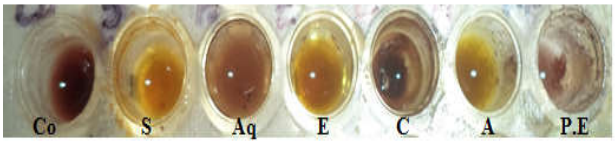

Figure 2 Qualitative ANALYSIS of anti-oxidant activity in *Hybanthus Enneaspermus* leaf extract with different solvents

Table 2 shows the free radical scavenging activity of *H. enneaspermus* leaf. Among all five extracts ethanol extract shows maximum free radical quenching property of DPPH. So ethanol extract taken for further analysis. Table.3 and Table.4 shows the presences and the concentration of primary metabolites and the secondary metabolites in *H. enneaspermus* leaf extract respectively.

other molecules (Sabnis and Daniel, 1990). Proteins are the primary components of living things. The presence of higher protein level in the plant points towards their possible increase food value or that a protein base bioactive compound could also be isolated in future (Thomsen *et al*., 1991).

Lipids are important component of biological membranes, free molecules and metabolic regulators that control cellular function and homeostasis (Chiang, 2005). The higher amount of plant lipid can be used as essential oils, natural food colors and spice oleoresins. With a strong foundation in research and development, plant lipids have developed products that work with diverse requirements, be it culinary, medicinal or cosmetic (Yadav and Tyagi, 2006).

In the present study the secondary metabolities like tannin, flavonoids, phenols, steroids, saponins, terpenoids and alkaloids were qualitatively analyzed by standard method. Tannin is phenolic compounds of high molecular weight used as antiseptic and this activity is due to presence of the phenolic group. This is also associated with antiviral activity. In presence of very high quantity of tannin, there are many viruses like polio virus, herpes simplex viruses have been found to get inactivated (Bajij, 1988).

Table 2 Quantitative analysis of antioxidant activity in the leaf extracts of *Hybanthus Enneaspermus*

	TIME (Minutes) Activity in %						
Samples	**0**	**5**	**10**	**15**	**20**	**25**	**30**
Standard (BHT)	87.448±0.524	88.624±0.569	92.659±0.622	95.711±0.680	96.705±0.523	97.167±0.271	98.302±0.477
Aqueous	72.436±0.745	74.724±0.532	75.712±0.577	76.825±0.588	77.278±0.656	77.851±0.794	79.510±0.856
Ethanol *	78.642±0.801	79.749±0.647	80.953±0.770	82.543±0.809	83.193±0.761	85.595±0.753	*87.421±0.764
Acetone	70.761±0.505	72.738±0.875	73.147±0.615	75.153±0.541	77.759±0.740	80.053±0.733	81.129±0.574
Chloroform	48.491±0.765	49.884±0.669	50.384±0.842	50.709±0.891	51.396±0.829	51.750±0.792	52.305±0.551
Petroleum Ether	**44.489±0.781	45.839±0.651	47.901±0.521	49.251±0.812	50.493±0.624	50.975±0.767	51.864±0.498

Each value is mean ± S.D. of 5 samples expressed as percentage of antioxidant activity.All values in the table significantly differs with experimental control at , BHT vs Ethanol *P < 0.01 level of significance and BHT vs Petroleum ether **P>0.01 level insignificance.

Table 3 quantification of primary metabolites In the leaf extract of *hybanthus enneaspermus*

S. No	Primary Metabolites	Quantity (mg/g)
1.	Carbohydrate	**86.340**±0.827
2.	Protein	**25.640**±0.595
3.	Lipids	**2.344**±0.754

Each value represents mean ± SD of 3 samples

Table 4 Quantification of Secondary Metabolites In The Leaf Extract of *Hybanthus Enneaspermus*

S.No	Secondary metabolites	Quantity
1.	Phenols	32.657±0.770 (mg GAE/g)
2.	Flavonoids	7.979±0.539 (mg QE/g)
3.	Alkaloids	10.80±0.563%
4.	Terpenoids	8.79±0.752%
5.	Tannins	19.883±0.469 (mg TAE/g)
6.	Steroids	15.903±0.515 mg/g
7.	Saponins	5.18 %

Each value represents mean ± SD of 3 samples

A primary metabolite is directly involved in the normal growth, development, and reproduction. Plants are rich sources of high value metabolites like carbohydrates, proteins and lipids are useful in flavoring, fragrances, insecticides, sweeteners and natural dyes (Kaufman *et al*., 1999).

Carbohydrates considered as an important compound which involves in production of many biochemical compounds. Carbohydrates perform numerous roles in living things includes storage of food (Dyke, 1960). Sometimes physical and chemical characters are modified by combining with

Tannin acts as primary antioxidants and possesses anti-inflammatory, antimicrobial, anticancer and anti-allergic activity (Rievere, 2009). Tannin have several physiological effects, such as to accelerate blood clotting, decrease the serum lipid level, reduce blood pressure, antimicrobial and anti-cancerous properties (Chang, 2015). Flavonoids are also responsible for the stimulation of antioxidant enzymes (Soto *et al*., 2003). Phenolic compounds are some of the most widespread molecules among plant secondary metabolites, are known to act as natural antioxidants (Jones *et al*., 1994; Skerget *et al*., 2005). Naturally occurring plant phenols and flavonoids possess a broad range of pharmacological activities such as antioxidant, antimutagenic, antimicrobial, antiulcer, antiarthritic, anti-cancer and protein kinase inhibition (Marinova *et al*., 2005; Sulaiman and Balachandran, 2012). Saponins are extensively utilized in veterinary vaccines because their character as an adjuvant and helps in the improvement of immune response (Parekh and Chands, 2007).

CONCLUSION

The antioxidant properties were analysed for different leaf extracts of *H. enneaspermus* using various solvents, ethanol was found to have the highest free radical quenching properties and the petroleum ether extract was found to the least properties. The leaf extract of *H. enneaspermus* when analyzed for presences of primary and secondary metabolites, phenolic compounds were found to be high.

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