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Phytochemical Analysis of *Enhydra Fluctuans* (Fam. – Asteraceae) Extracts to Detect Their Chemical Components With Antioxidant Property

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Abstract : Antioxidants are the substances that inhibit oxidation and have the ability to remove the potentially damaging oxidizing agents in a living organism. Phytochemicals like flavonoids and phenols present in plants are able to reduce or prevent the oxidative damage to the human cells. *Enhydra fluctuans* Lour. (Fam. Asteraceae) is a marshy herb used by many tribal communities of North East India as well as in traditional medicine for treatment of various ailments. In the present study, ethanolic and aqueous extract of this plant were tested for Phytochemical and antioxidant activities. Phytochemical screening of the extracts done by routine analytical procedures revealed the presence of saponin, terpenoids, phenol and proteins in both the extracts. However, alkaloids, and tannins which were found to be present in the ethanolic extract were found to be absent in the aqueous extract of *E. fluctuans*. Quantitative determination of total phenolics and *in-vitro* DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical Scavenging activity of both the extracts were carried out using spectrophotometric analysis. The total Phenolic content was found to be 60.67±0.083µg/ml GAE and 39.83±0.083 µg/ml GAE for ethanolic and aqueous extract, respectively. They exhibited strong antioxidant DPPH radical scavenging activity with IC50 value of 1.38µg/ml, 43.65µg/ml and 158.49µg/ml for ascorbic acid, aqueous extract and ethanolic extract respectively. The result of present comprehensive analysis demonstrated that *E. fluctuans* possesses potential antioxidant activity, and could be used as a viable source of natural antioxidants and might be exploited for pharmaceutical applications.

Keywords: Antioxidants, DPPH, *Enhydra Fluctuans,* Free radicals, Phytochemical screening, total phenol content.

Introduction

Since ancient times, people have been using plants as an origin of drugs to treat various diseases. Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts (Sandhya *et. al*., 2011). The plant kingdom harbours enormous reservoir of biologically active compounds called phytochemicals with various chemical structures and protective / disease preventing properties. Scientists estimate that there may be as many as 10,000 different phytochemicals with the potential to affect disease such as cancer, stroke or metabolic syndrome.

Therefore, phytochemical screening is very important in identifying new sources of therapeutically and industrially important secondary metabolites like alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc. (Akindele *et. al*., 2007).

Reactive oxygen species (ROS) exert oxidative damaging effects to cell components and molecules (Bansal *et. al*., 2011). Damage to DNA, proteins and other macromolecules due to oxidation has been implicated in the pathogenesis of a wide variety of degenerative diseases, most notably cancer and heart disease (Halliwell *et. al*., 1994). Antioxidants can help prevent oxidative damage induced by free radicals. However commercially available synthetic antioxidant agents like butylated hydroxyanisole (BHT) have been reported to be toxic to animals including human beings which have stimulated the interest of many researchers to search natural antioxidants (Vinay *et. al*., 2010) . Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of diseases (Shekhar *et. al*., 2011). It has been reported that antioxidant activity of plants might be due to their flavonoids and phenolic compounds (Cook and Samman, 1996; Samantha *et. al*., 2012). Plant phenolics are widely distributed in the tissues of plants and play a very important role in free radical scavenging due to their hydroxyl groups.

Enhydra fluctuans, commonly known as water crest or Marsh herb, belonging to the family Asteraceae, is a hydrophytic plant. In India, this plant is predominantly found in the North-Eastern region and mostly in Assam (Chakraborty *et. al*., 2012). *E. fluctuans* is used by the tribal communities for treatment of various diseases. It has many beneficial effects such as anti-inflammatory activity (Sannigrahi *et. al*., 2010); antioxidant activity (Uddin *et. al*., 2005); anti-cancer activity (Kumar *et. al*., 2012); anti-diarrhoeal activity (Kumar and Khanum, 2012); hepatoprotective activity (Patil KS *et. al*., 2008); analgesic activity; neuroprotective potential (Alebiosu *et. al*., 2015); Phagocytic and cytotoxic activity (Hassan *et. al*., 2015).

This study has been undertaken to determine the phytochemicals present, total Phenolic content and the antioxidant property of ethanolic and aqueous extract of *Enhydra fluctuans* by DPPH free radical scavenging method.

Materials and Methods

Collection f Plant Materials

Fresh leaves and stems of *Enhydra fluctuans* were collected in August from local area of Guwahati. The plant material collected was authenticated(**Ref. no. Herb./Bot./GU/2016/144)** in the Department of Botany, Gauhati University, Guwahati.

Preparation of Crude Ethanolic Extract

The leaves and stems were washed carefully with water and shade dried at room temperature and then ground into a coarse powder with the help of a suitable grinder. The powders were stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

About 100g of powdered material was taken in a clean, flat-bottomed glass container and soaked in 500ml of 95% ethanol. The container with its contents was sealed and kept for a period of 10 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper. The

filtrates so obtained were allowed to evaporate under normal temperature. Gummy concentrate of greenish and brownish colour was obtained which was designated as ethanol extract of *E. fluctuans* (EEEF), dried in vacuum evaporator and stored under 4°C until further investigation (Islam *et. al*., 2011).

Preparation of Aqueous Extract

About 27g powder was macerated with distilled water and kept for a period of 3 days with occasional stirring. The mixture was filtered using whattman filter paper and the filtrate was lyophilized. A brownish colored aqueous extract of *E. fluctuans* (AEEF) was obtained that was stored under 4°C until further investigation (Nabi *et. al*., 2013; Semwal *et . al*., 2012)

Qualitative Analysis (Tests for Chemical Group)

EEEF and AEEF were subjected to preliminary quantitative phytochemical investigation for the detection of some important secondary metabolites (Ncube *et. al*., 2008; Sandhya *et. al*., 2011)

1. Test for alkaloids

0.2g of the extracts were heated on a boiling water bath with 2N HCl (5ml). After cooling, the mixtures were filtered.

I. **Mayer's Test**: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of yellow coloured precipitate indicated the presence of alkaloids.
II. **Wagner's Test**: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicated the presence of alkaloids.

2. Test for glycosides

1.2g of the extracts were hydrolyzed by 10ml of 1% HCl solution and neutralized with 10% of NaOH solution. A few drops of Fehling's solution A and B were added. The formation of red precipitate indicated the presence of glycosides.

3. Test for tannins (Ferric Chloride Test)

0.2g of each extracts were mixed with 10ml of distilled water and heated. The mixtures were filtered and to each filtrate 5% (w/v) solution of ferric chloride were added and the formation of dark green solution indicated the presence of tannins.

4. Test for flavonoids (Alkaline reagent Test)

0.2g of each extracts were taken and dissolved in diluted NaOH and 1M of HCl was (5ml each) added. A yellow solution that turns to colorless indicated the presence of flavonoids.

5. Test for saponins (Froth Test)

0.2g of each extracts was diluted each with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1cm layer of foam indicated the presence of saponins.

6. Test for terpenoids

0.2g of each extracts were mixed separately with 2ml of chloroform and concentrated sulphuric acid (3ml) and then added carefully, to form a layer. A formation of reddish brown coloration of the solution at inert face indicated the presence of terpenes.

7. Test for phenols (Ferric Chloride Test)

About 0.2g of each extracts was dissolved in a mixture of water and ethanol, and a few drops (3-4) ferric chloride solution was added. Red, blue, green or purple coloration indicated presence of phenols.

Quantitative analysis of total phenol content using the Folin-Ciocalteu reagent assay adapted by Singloten and Rossi, 1965.

10mg of gallic acid was dissolved in 100ml of 50% ethanol (100µg/ml). Further diluted to 20, 40, 60 1nd 80µg/ml. 1ml of each dilution was taken in a test tube. Concentration of 1mg/ml of plant extract was prepared in ethanol. 1ml of each sample were taken in test tubes. 1.5ml Folin-Ciocalteu reagent was added to each test tubes. Incubated at room temperature for 8 mins for reactions to take place. 2ml of 20% Na2CO3 was added to each test tubes. Mixed well. Test tubes allowed to stand for 1 hour at room temperature. Absorbance of the standard measured at 760nm. Blank : 1ml 50% methanol, 1.5ml FCR and 2ml Na2CO3 solution (Singleton and Rossi, 1965).

Evaluation of antioxidant activity by DPPH radical scavenging method

DPPH is a stable, nitrogen centered free radical which produces deep purple colour in ethanol solution. The principle of this assay is based on the reduction of purple colored ethanolic DPPH solution in the presence of hydrogen donating antioxidants by the formation of yellow colored diphenyl-picryl hydrazine. Lower absorbance of the reaction mixture indicated higher free radical activity. This reaction has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the anti-oxidative activity of foods and plant extracts (Zhou *et. al*., 2004; Panovska *et. al*., 2005).

0.1mM solution of DPPH in ethanol was prepared. This solution (1 ml) was added to 3 ml. of EEEF and AEEF extracts in ethanol at different concentration (1, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml). The mixture was shaken vigorously and allowed to stand at room temp for 30 min. then, absorbance was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu) (Ahmed *et. al*., 2013). Absolute alcohol was used as blank. Reference standard compound being used was ascorbic acid and experiment was done in triplicate (Patel *et. al*., 2011). The IC 50 value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity (Koleva *et. al*., 2002). The percent DPPH scavenging effect was calculated by using following equation:

$$\text{DPPH scavenging effect (\%) or Percent inhibition} = A0 - A1 / A0 \times 100.$$

Where A0 = the Absorbance of control reaction
And A1 = the Absorbance in presence of test or standard sample (Achola *et al*., 1998).

Results

Percentage yield of plant extract.

The percentage yield of EEEF and AEEF is shown below (Table 1)

Table 1 : Percentage yield of plant extract

Sl. No.	Extracts	Color of the extracts	Yield of extracts (g)	Yeild of extracts (%)
1	EEEF	Dark green	5.46	3.64
2	AEEF	Dark brown	2.32	8.59

Photoplate 1. Extracts of E. fluctuans –
A. Ethanolic extract B. Aqueous extract

Phytochemical Screening of plant extracts

The Phytochemical screening of EEEF revealed the presence of alkaloids, tannins, saponins, terpenes, phenols and proteins while AEEF revealed the presence of saponins, terpenes, carbohydrates, phenols and proteins. Flavonoids and glycosides were found to be absent in both the extracts (Table 2).

Table2. Phytochemical screening of EEEF and AEEF.

Chemical components	EEEF	AEEF
Alkaloids	+	-
Glycosides	-	-
Tannins	+	-
Flavonoids	-	-
Saponins	+	+
Terpenes	+	+
Carbohydrates	-	+
Phenols	+	+
Proteins	+	+

Present (+) ; Absent (-)

Quantitative Analysis of Total Phenolic Content (Tpc) of Eeef and Aeef

Using the calibration curve of Gallic acid (Fig.1), TPC in the EEEF was found to be 60.67µg/ml GAE and in AEEF 39.8367µg/ml GAE, respectively (Table. 3). TPC was found to be higher in EEEF than in AEEF.

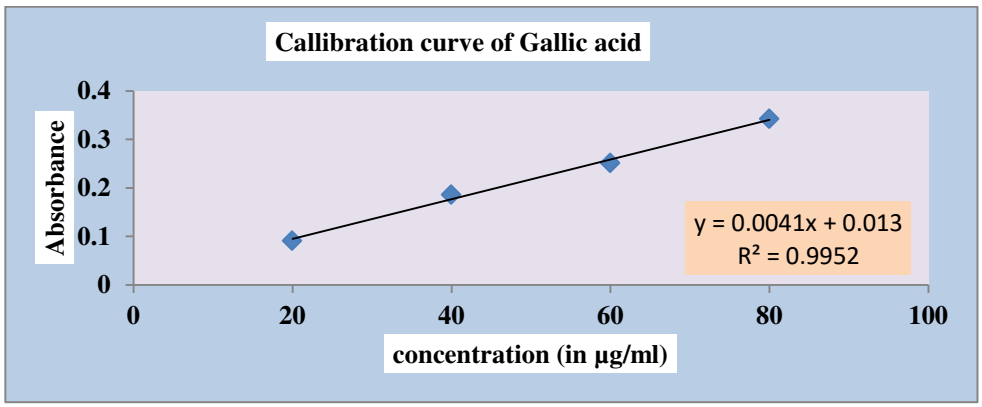

Fig 1. Calibration curve of Gallic acid for TPC determination.

Table 3. Total Phenolic content (TPC) as Gallic acid Equivalent

Sl. No.	Extract	TPC as Gallic acid equivalent (in µg/ml)
1.	AEEF	39.83±0.083
2.	EEEF	60.67±0.083

DPPH Free Radical Scavenging activity of EEEF and AEEF

As shown in table 4, DPPH decolorization was increased by the *E. fluctuans* extracts in a concentration dependent manner. Both the extracts are good scavengers of DPPH free radical. AEEF at a concentration of 100µg/ml was able to scavenge 54.83±0.07% of DPPH free radical while EEEF was able to scavenge 42.32±0.009% of DPPH free radical. Thus, AEEF showed a better free radical scavenging activity. Also, IC_{50} value of AEEF (43.65µg/ml) is lower than that of the EEEF (158.49µg/ml) that indicates towards higher activity of AEEF (Table 4)

Table 4 : DPPH Free Radical Scavenging potential of EEEF, AEEF and Ascorbic acid. Values are represented as mean± SEM

Concentration (in µg/ml)	% inhibition		
	EEEF	**AEEF**	**Ascorbic acid**
10	26.73 ± 0.03	40.07 ± 0.03	79.1 ± 0
20	27.43 ±0.01	41.6 ± 0	80.6 ± 0
30	32.53 ± 0.03	48.97 ± 0.03	88 ± 0.058
40	34.63 ± 0.01	51.93 ± 0.03	90.9 ± 0.033
50	34.70 ± 0.01	52.2 ± 0.15	91.2 ± 0.145
60	37.96 ± 0.003	52.4 ± 0.12	91.4 ± 0.115
70	38.63 ± 0.03	52.2 ± 0.17	91.7 ± 0.173
80	40.6 ± 0.006	53.67 ± 0.17	92.6 ± 0.167
90	41.27 ± 0.006	53.97 ± 0.07	92.9 ±0.067
100	42.32 ± 0.009	54.83 ± 0.07	93.5 ± 0.067
IC50	**158.49µg/ml**	**43.65 µg/ml**	**1.38 µg/ml**

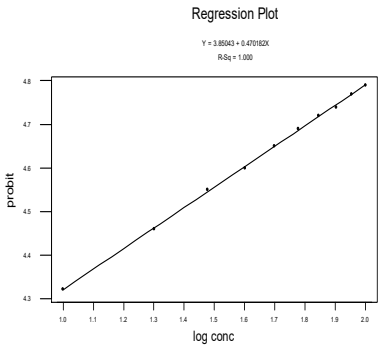

Fig 2. Regression graph for EEEF

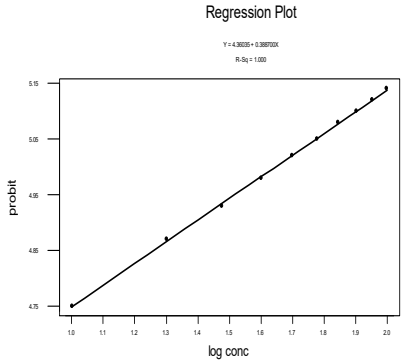

Fig 3. Regression graph for AEEF

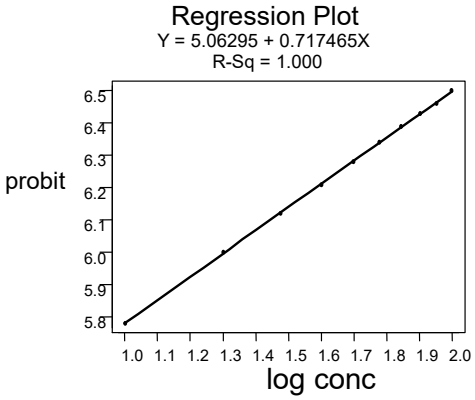

Fig.4 Regression graph for Ascorbic acid

Discussion

Result from the present investigation shows that *E. fluctuans* is rich in Phytochemicals. Phytochemical screening of Ethanolic extract of *Enhydra fluctuans* (EEEF) revealed the presence of alkaloids, tannins, saponins, terpenoids, and phenols. Till now, there has been no report on the phytochemical analysis of ethanolic extract of *E. fluctuans*. However, methanolic extract of *E. fluctuans* has been screened for Phytochemical constituents and workers reported the presence of tannin, saponin and absence of terpenoids, flavonoids, alkaloids, glycoside (Panigrahi et. al., 2015) which is in agreement to our findings. Some other workers (Kuri et. al., 2014) reported the presence of flavonoids, triterpenes, carbohydrates, reducing sugar, saponins, phenols, diterpenes, proteins and tannins and absence of alkaloid, cardiac glycoside and phytosterols. On the other hand, the AEEF showed the presence of saponins, terpenes, carbohydrates and phenols. However, Phytochemical analysis of AEEF by Dua et. al., 2016 revealed the presence of flavonoids, phenols, carbohydrates, glycosides, alkaloids, tannins and amino acids (Dua et. al., 2016). The difference in results may be due to differences in area of plant collection (Ghasemzadeh et. al., 2018), choice of solvent and method of extraction (Simon et. al., 2015).

Plant phnolics are widely distributed in the tissues of plants and play a very important role in free radical scavenging due to their hydroxyl groups. The total Phenolic content in the AEEF and EEEF were found to be 39.83±0.083 µg/mlGAE and 60.67±0.083 µg/mlGAE, respectively. EEEF has shown higher amount of TPC which is similar to the findings of Pramod et. al., 2012, where the EEEF showed higher content of phenol compared to pet-ether and chloroform extracts, and Dua et. al., 2016, wherein TPC of AEEF was found to be ~21.3mg/g^{DW} pyrocatechol equivalent. (Dua et. al., 2016; Swain et. al., 2012)

In the present study, IC_{50} of EEEF, AEEF and Ascorbic acid were found to be 158.49µg/ml, 43.65µg/ml and 1.38µg/ml, respectively at a concentration of 100µg/ml. Both the extracts have shown potent antioxidant activity as compared to ascorbic acid which is in agreement with the works done by Swain et. al., 2012 and Dua et. al., 2016.. However, there has been no report on the DPPH free radical scavenging activity of AEEF, but, AEEF (400 µg/ml) significantly returned the $NaAsO_2$-induced alteration of lipid peroxidation, protein carbonylation, and antioxidant markers to close to untreated levels (Dua et. al., 2016). Our results suggests that AEEF showed higher free radical scavenging activity in a concentration dependent manner. In spite of having a lower TPC than EEEF, AEEF exhibited a higher free radical scavenging activity. This may be due to the antioxidant activity of other active phytoconstituents present in AEEF. This fact can be validated by the findings of Ghosh et. al., 2013, wherein biochemical analysis of a water extracted carbohydrate polymer of *E. fluctuans* containing esterified Phenolic acids, revealed similarity in antioxidant potential when compared with known standard antioxidant. Thus, our findings represent an interesting approach in phytotherapeutic treatments that might include the use of the aqueous extract of *E fluctuans* as a possible new source of natural antioxidant to combat diseases like cancer, diabetes, etc. caused by oxidative stress.

Conclusion

From this study, we can conclude that the AEEF was found to exhibit higher free radical scavenging activity and could be investigated as a possible new source of natural antioxidants in the food and pharmaceutical industry. This may also lead to the development of a new generation of drugs that possess both chemotherapeutic and chemo protective properties which can result in ways of combating the serious problems of diseases including diabetes.

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