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Evaluation of Antioxidant Activity of *Diplazium esculentum* and *Enhydra fluctuans* of West Bengal

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Abstract: *Reactive oxygen species mediated cell damage is one of the main causes of many diseases and hence natural antioxidants have their own benefits on human health. The present study was carried out to investigate the antioxidant potential of aqueous ethanolic extract of different parts i.e. leaves, stems and roots of Diplazium esculentum and Enhydra fluctuans, two well known and widely consumed leafy vegetables in India. The antioxidant potential of these two plants was examined by DPPH radical scavenging assay, ABTS·+ radical scavenging assay, Hydroxyl radical scavenging assay and by determination of total phenolic and flavonoid content. It was found that the leaves of Enhydra fluctuans (known as Helencha or Hinche in Bengali) and Diplazium esculentum (known as Dheki in Bengali) exhibit a significant amount of antioxidant potential than the respective stems and roots. It can be concluded that among the two plants the antioxidant activity follow the sequence Enhydra fluctuans>Diplazium esculentum. The evidences can be concluded from the study of the aqueous ethanolic extract of different parts of Helencha and Dheki impose that the two can be used as a source of potent natural antioxidants.*

Keywords: *Enhydra fluctuans; Diplazium esculentum; Helencha; Dheki; Antioxidant; Polyphenols.*

I. Introduction

Many people of developing and underdeveloped countries around the world depend on traditional sources of medicines like herbs and plants as the only affordable remedy against diseases till date [1, 2]. Not only that, nowadays also in developed countries, natural herbal medicines are prescribed in case of chronic diseases as to avoid the harmful side effects of prolonged use of chemical synthetic drugs [3]. The reasons of the worldwide growing popularity of natural medicines are their effectiveness, lesser side effects, easy availability and low cost.

In India, since the ancient era, many greens are used both as medicine and as vegetable in daily life. This study focused on two medicinal and edible greens easily available and widely used as vegetables in West Bengal, India namely ***Diplazium esculentum (Retz.) [Dheki]*** and ***Enhydra fluctuans (Lour.) [Helencha].*** Besides consuming as vegetable, *D. esculentum* is used in traditional medicine in diseases like fever, dermatitis, measles [4], dysentery, glandular dwellings, indigestion, diarrhea and various skin infections [5] and *E. fluctuans* have a wide use as a folk remedy in conditions like gastric ulcer [6], inflammation, skin diseases and small pox [7].

The greens are consumed in West Bengal over hundreds of years with no known harmful side effects. The objective of the present study is to evaluate the antioxidant properties of ***Diplazium esculentum (Retz.)[Dheki]*** and ***Enhydra fluctuans (Lour.)[Helencha]*** plants using in vitro models.

II. Materials and Methods

A. Materials

1. Chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Himedia, India. 2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Sigma, USA. All other chemicals used in the research including ethanol, gallic acid, sodium carbonate, folin-ciocalteu, NaOH, $NaNO_2$, $AlCl_3$, $FeSO_4$, EDTA, potassium persulfate, ascorbate, quercetin, 2-Deoxyribose sugar, H_2O_2, TCA, TBA were of analytical grade procured from Merck, India, SRL, India and Universal, India. Deionized distilled water was used in the entire study.

2. Plants

Fresh and healthy plants of two species i.e. *Enhydra fluctuans (Helencha) and Diplazium esculentum (Dheki)* were purchased from the local market at Shyamnagar, North 24 Parganas, West Bengal during April, 2015. Fresh leaves of uniform shape and color were selected while diseased leaves were eliminated.

B. Methods

1. Extraction of plants

At first, the leaves, stems and roots of both plants were separated from each other and washed thoroughly for several times with distilled water until no foreign material remains in it. Then the samples were air-dried for 24 hours and after that they were oven-dried at 70°C for 72 hours. Next they were pulverized to powder with a Waring blender. Aqueous ethanolic extracts of different plant parts were prepared by suspending the powder (0.25 gm) of each part of the two plants in 10 ml 60% aqueous ethanol. The mixtures were heated for 1 hour in a 90°C water bath. After that, the extracts were vacuum filtered and the resulting filtrates were centrifuged at 7830 rpm at 4°C for 5 minutes. The supernatant obtained were freeze-dried to constant weight and extract yield were recorded. The freeze-dried extracts were then redissolved in 60% aqueous ethanol and then stored at -20°C until further use.

2. Total Phenolic Content (TPC) Assay

The total phenolic contents of the test samples were determined spectrophotometrically using Folin-Ciocalteu reagent by applying a method previously described [8] with some modification. To 300 µl of test samples (60% aqueous ethanolic extract of each sample, in a 1:10 dilution), 1000 µl of Folin-Ciocalteu reagent (1:20 dilution) and 300 µl sodium carbonate (7.5%) were added and incubated at room temperature for 60 minutes. The color developed was measured at 765nm using a UV-Vis spectrophotometer (Systronics, India, Model-2202). TPC was expressed as gallic acid equivalent (GAE) per gm dried sample weight.

3. Total Flavonoid Content (TFC) Assay

The total flavonoid contents of the test samples were measured following a standard spectrophotometric method [9]. In a 10 ml volumetric flask, one ml of aqueous ethanolic extract of samples was diluted with 4 ml of distilled water with the addition of 0.3 ml of 5% $NaNO_2$ solution. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added and then after 6 minutes, 2 ml of 1M NaOH was added in each volumetric flask. Then, 2.4 ml of distilled water was added to the reaction flask and mixed well. Absorbance of the reaction mixture was measured spectrophotometrically by a UV-Vis spectrophotometer (Systronics, India, Model-2202) at 510 nm. The total flavonoid content in different extracts was calculated as quercetin equivalent (QE) per gm dried sample weight.

4. Antioxidant activity assay by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity

The antioxidant activity of test samples were measured according to a method previously described [10] in terms of hydrogen donating or radical scavenging ability using the stable DPPH method with some modification. The 1 ml reaction mixture contained 250 µl ethanolic DPPH, 450 µl ethanol and 300 µl 60% aqueous ethanolic extract (1:10 dilution) of two plant samples. In the control preparation 750 µl aqueous ethanol was used in place of sample. The contents were mixed well and incubated for 20 minutes in dark place at room temperature. The degree of reduction of absorbance was recorded in a UV-Vis spectrophotometer (Systronics, India, Model-2202) at 517 nm. DPPH scavenging activity was expressed as gallic acid equivalent (GAE) per gm dried sample weight.

5. Antioxidant activity assay by 2,2'-Azinobis-(3-Ethylbenzothiazoline-6-sulfonic acid (ABTS·+) radical scavenging capacity

Antioxidant capacity was also measured based on ABTS·+ radical scavenging capacity according to a method previously described [11]. The ABTS solution was mixed with potassium persulfate and incubated for 12-16 hours in dark to generate ABTS·+ cation. Then 20 µl sample solution (60% aqueous ethanolic extract in 1:10 dilution) was mixed with 980 µl ABTS·+ solution. In the control preparation 20 µl aqueous ethanol was used in place of sample. The contents were mixed well immediately and incubated for 5 minutes in a dark place. The absorbance was measured at 534nm in a UV-Vis spectrophotometer (Systronics, India, Model-2202). ABTS·+ scavenging activity was expressed as gallic acid equivalent (GAE) per gm dried sample weight.

6. Antioxidant activity assay by hydroxyl radical scavenging capacity

The scavenging assay of hydroxyl radical was done according to a method previously described [11]. In this assay, at first hydroxyl radical was produced by the Fenton reaction through $Fe3^{+}$-ascorbate-EDTA-H_2O_2 system. In that system 60% aqueous ethanolic extracts of two plants in 1:10 dilution was added which quench the hydroxyl radical. The remaining hydroxyl radicals degrade the 2-deoxyribose sugar to malondialdehyde (MDA) which condensed with TBA to form a pink chromogen. Then it was measured spectrophotometrically by

UV-Vis spectrophotometer (Systronics, India, Model-2202) at 532 nm against appropriate blank. Hydroxyl radical scavenging activity was expressed as gallic acid equivalent (GAE) per gm dried sample weight.

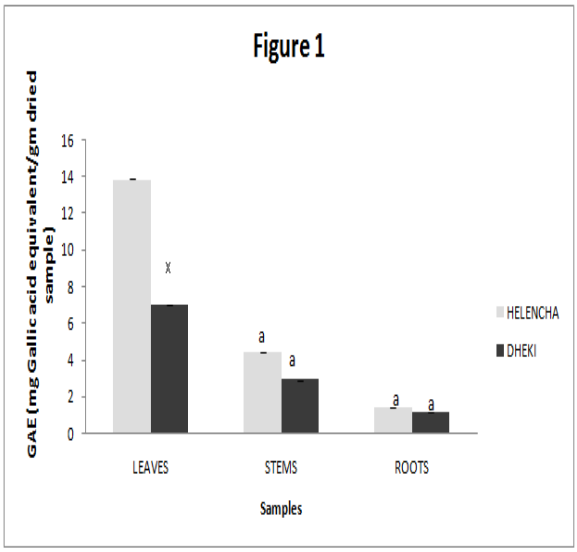
III. Statistical Analysis

All the analysis were carried out in triplicate (n=3) and the results were expressed as mean ± SEM. One way ANOVA (Analysis of variance) with Tukey's test were performed to determine significant differences between means where applicable ($p<0.05$ was considered as statistically significant).

IV. Result and discussion

A. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of samples

Phenolic and flavonoid contents are very important plant constituents because of their scavenging ability due to the presence of their hydroxyl groups [12] and so they serve as significant indicators of potential antioxidant activity. It's effects on human nutrition and health is also considerable. The phenolic contents are most widely present phytochemicals in plants which act as antioxidant and thus reduce the risk of oxidative stress induced diseases [13-16]. **Fig. 1** indicates the phenolic contents in the aqueous ethanol extracts of leaves of *Enhydra fluctuans* (helencha) (13.88 ± 0.002 mg GAE/gm dried leaves) and *Diplazium esculentum* (dheki) (7.01 ± 0.002 mg GAE/gm dried leaves) are significantly high with respect to their corresponding stem (Helencha 216.17 %, $p<0.001$ and Dheki 140.9%, $p<0.001$) and root extracts (Helencha 898.6 %, $p<0.001$ and Dheki 504.31 %, $p<0.001$) under similar experimental conditions (**Fig. 1)**.



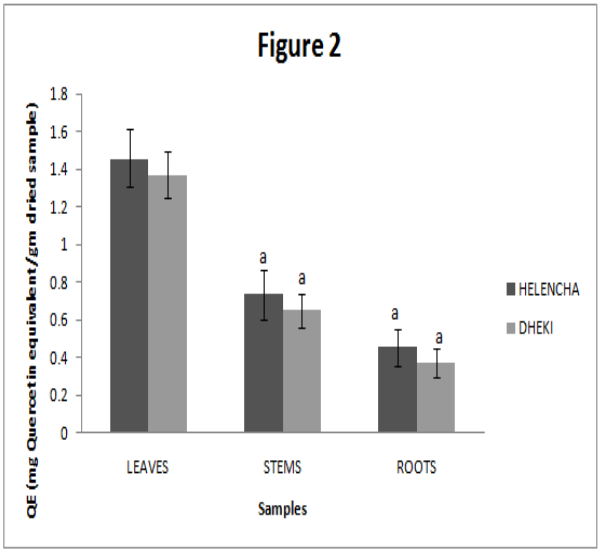


Fig.1: Total phenolic content of aqueous ethanolic extracts of leaves, stems and roots of *Enhydra fluctuans* (Helencha) and *Diplazium esculentum* (Dheki). Data points are mean ± SEM (n= 3). Significant difference from corresponding leaves extracts $^{a}p<0.001$. Significant difference from corresponding *Enhydra fluctuans* ($^{x}p<0.001$) leaves extract

Fig.2: Total flavonoid content of aqueous ethanolic extracts of leaves, stems and roots of *Enhydra fluctuans* (Helencha) and *Diplazium esculentum* (Dheki). Data points are mean ± SEM (n= 3). Significant difference from corresponding leaves extracts $^{a}p<0.001$.

Fig. 2 represents the flavonoid contents in aqueous ethanol extracts of leaves of helencha (1.46 ± 0.15 mg QE/gm dried leaves) and Dheki (1.37 ± 0.12 mg QE/gm of dried leaves) which are also significantly high with respect to their corresponding stem (Helencha 100%, $p<0.001$ and Dheki 110.77%, $p<0.001$) and root extracts (Helencha 224.44%, $p<0.001$ and Dheki 270.27 %, $p<0.001$) under similar experimental conditions (**Fig. 2)**.

The phenolic compounds are produced in the plants as a defense mechanism to combat different environmental stress [17-21]. Phenolic and flavonoid compounds provide protection against UV-B mediated cell

damage and subsequent cell death by protecting DNA from dimerization and breakage [22]. Environmental factor such as light also stimulates the synthesis of flavonoids, especially anthocyanins and flavones via phenylalanine ammonia lyase (PAL) [23]. This may explain the presence of greater amount of phenolic and flavonoid content in leaves than in stems and roots as the leaves are more vulnerable to environmental stress than the other parts. The natural habitats for both plants are moist or wet and shady areas such as riverbank, along the sides of ponds and in rice fields etc. The high phenolic and flavonoid content of the plants are could be due to their tolerance to such adverse environment.

B. Antioxidant activity

1. DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay

DPPH is a common abbreviation for an organic chemical compound 1, 1-diphenyl-2-picrylhydrazyl. It is a dark-colored crystalline powder composed of stable free-radical molecules. DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. It is a sensitive way to survey the antioxidant activity of a specific compound or plant extracts [24]. The antioxidant activity is directly proportional to the fading of the purple color in the DPPH test samples.

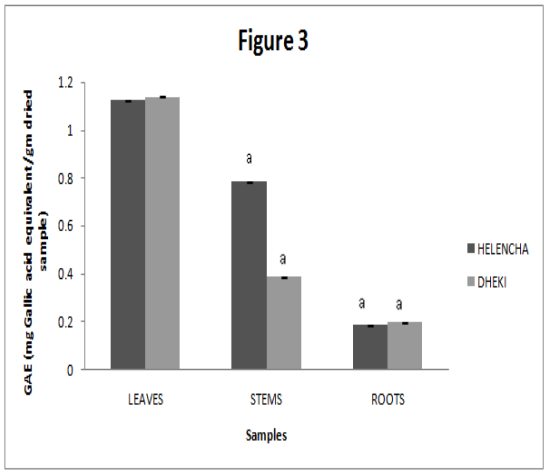


Fig.3: DPPH Scavenging assay of aqueous ethanol extracts of leaves, stems and roots of *Enhydra fluctuans* (Helencha) *and Diplazium esculentum* (Dheki). Data points are mean ± SEM (n= 3). Significant difference from corresponding leaves extracts [a]p<0.001.

Fig. 3 depicts DPPH radial scavenging activity of aqueous ethanol extracts of leaves of helencha (1.125±0.003mg GAE/gm dried leaves) and dheki (1.138 ± 0.003mg GAE/gm dried leaves) along with their stems and roots and both of these two leaves maintain a significantly high DPPH radial scavenging activity compare to their corresponding stem (Helencha 43.31%, p<0.001 and Dheki 195.58%, p<0.001) and root extracts (helencha 514.75%, p<0.001 and dheki 480.61%, p<0.001) under similar experimental conditions (**Fig 3**). Therefore, both dheki and helencha, particularly their leaves maintain a significant high radical scavenging activity. It may be due to the hydroxyl groups present in the chemical structure of phenolic compounds that can provide the necessary component as a radical scavenger and thus provide a promising source of nutraceutical supplements.

2. ABTS·+ (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

ABTS·+ is a blue colored chromophore which is reduced to ABTS on a concentration dependant manner upon addition of the plant extract [11]. ABTS·+ assay is more versatile as both the polar and non-polar samples can be evaluated for their scavenging activity. **Fig.4** represents a ABTS·+ radical scavenging activity of aqueous ethanol extracts of leaves of helencha (11.56 ± 0.002mg GAE/gm dried leaves) and dheki (9.98 ± 0.002 mg GAE/gm dried leaves) which are significantly high with respect to their corresponding stem (Helencha 90.76%, p<0.001 and Dheki 149.5%, p<0.001) and root extracts (Helencha 480.90%, p<0.001 and Dheki 543.87%, p<0.001) under similar experimental conditions (**Fig.4**).

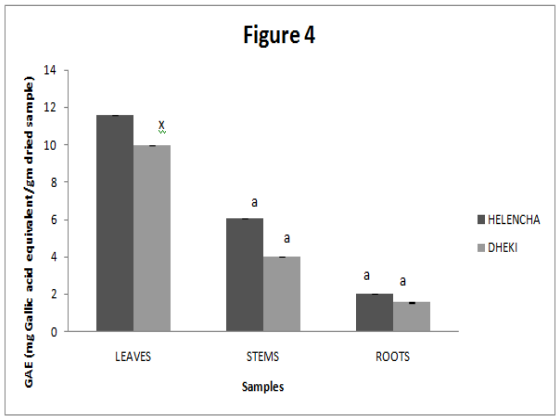


Fig.4: ABTS·+ Scavenging assay of aqueous ethanol extracts of leaves, stems and roots of *Enhydra fluctuans* (Helencha) *and Diplazium esculentum* (Dheki). Data points are mean ± SEM (n= 3). Significant difference from corresponding leaves extracts [a]p<0.001. Significant difference from corresponding *Enhydra fluctuans* ([x]p<0.001) leaves extracts.

3. Hydroxyl radical scavenging assay

Unlike DPPH and ABTS·+ radicals, which are less relevant to the biological systems, reactive oxygen species (ROS) are commonly found in living tissues. The most detrimental of the free radicals formed in biological systems by various reactions is the hydroxyl radical that causes enormous damages on biomolecules of the living cells [25]. As the extracts are added to the Fenton reaction

mixture the hydroxyl radicals are scavenged and thereby sugar damage can be blocked.

Fig. 5 represents hydroxyl radical scavenging activity of aqueous ethanol extracts of leaves of helencha (52.32 ± 0.002 mg GAE/gm dried leaves) and dheki (50.12 ± 0.001mg GAE/gm dried leaves) along with their roots and stems and hydroxyl radical scavenging activity of leaves are significantly high with respect to their corresponding stem (Helencha 315.57%, $p<0.001$ and Dheki 321.53%, $p<0.001$) and root extracts (Helencha 506.26 %, $p<0.001$ and Dheki 577.3 %, $p<0.001$) under similar experimental conditions (**Fig.5**).

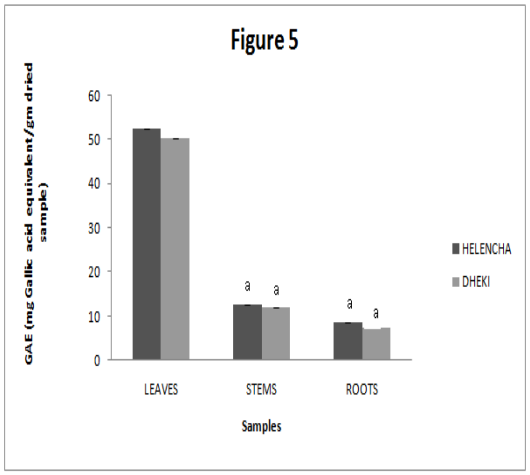


Fig.5: Hydroxyl radical scavenging assay of aqueous ethanol extracts of leaves, stems and roots of *Enhydra fluctuans* (Helencha) *and Diplazium esculentum* (Dheki). Data points are mean ± SEM (n= 3). Significant difference from corresponding leaves extracts $^{a}p<0.001$.

In this study, all the antioxidant activities were expressed as Gallic acid equivalents per gram of dried plant material as it is a more meaningful and descriptive expression than assays that express antioxidant activity as the percentage decrease in absorbance as such the results provide a direct comparison of the antioxidant activity with standard Gallic acid. The plants show a high level of antioxidant activity in means of DPPH, ABTS·+ and hydroxyl radical scavenging assay. In comparison to DPPH radical scavenging assay, ABTS·+ and hydroxyl radical scavenging assay show the opposite trend. But the leaves, in all cases, exhibit more potentiality as antioxidant than stems and roots (**Fig. 3, 4 and 5**).

V. Conclusion

Enhydra fluctuans (helencha) and *Diplazium esculentum* (dheki) are two very common green leafy vegetables which are broadly consumed by the people of West Bengal in daily life from the ancient era without properly knowing the proper benefits. They are popular in high as well as low income group of population as they are both very cheap and easily available. This study focuses on exploring the health benefits of these plants. The results obtained from the present study show that leaves from *Enhydra fluctuans* and *Diplazium esculentum* have appreciable amount of polyphenols and flavonoids and also exhibit strong radical scavenging activity. Since, the antioxidant activity of a drug may depend on the total polyphenol-flavonoid content and free radical scavenging activity [26], therefore these two leafy vegetables are safe to use as natural antioxidant supplements and may also provide a potent source of nutraceutical supplements to prevent the initiation of disease related with oxidative stress. Future studies can also be carried out to find the effect of these two plants against particular diseases such as diabetes mellitus, CHD etc. The specific compounds responsible for their antioxidant activity are needed to be found and further investigation by fractionation of the extracts and then analysis for active compounds responsible for these activities could be done in future. This might lead to the development of new drugs.

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