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Phytochemical Screening and Invitro Antioxidant Activity of *Limnathemum Indicum.*

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ABSTRACT

The present study was carried out to investigate the in-vitro activity of ethanolic extract of Whole plant of *Limnanthemu indicum* by using nitric oxide radical scavenging activity, ABTS radical scavenging activity and lipid peroxidation radical scavenging activity. The extract was also subjected for preliminary phytochemical analysis and for the estimation of total phenolic and flavanoid content using standard procedure. Result suggests that the extract possess mild antioxidant activity as compared to the standard ascorbic acid which can be attributed to the presence of lower concentration of total phenols and flavanoids. However, this plant needs to be evaluated for its antioxidant activity with other solvent extracts and further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract.

Keywords: Antioxidants, *Limnanthemu indicum*, ethanolic extract, lipid peroxidation, ABTS radical.

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INTRODUCTION

In our body as a result of biological oxidation, free radicals or ROS are formed. Excessive production of these free radicals contributes to oxidative stress [1,2], which leads to the damage of proteins, DNA and lipids that is associated with the chronic degenerative diseases including cancer, coronary artery disease, hypertension and diabetes etc [3].

The recent abundant evidence suggests that reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical, involve in the pathogenesis of various disorders and diseases [4]. Most ROS are scavenged by endogenous defense systems such as catalase, superoxide dismutase, peroxidase-glutatione system etc [5]. But these systems may not be completely efficient, making them to depend on exogenous antioxidants from natural resources.

Hence, the development of antioxidants from natural origin has attracted considerable attention and is thought to be a desirable development. Moreover, several studies have indicated that medicinal plants contain a wide variety of natural antioxidants such as phenolic acids, flavonoids and tannins, which possess antioxidant activity [6].

Limnanthemum indicum an aquatic floating herb belonging to the Menyanthaceae family commonly called as water snow flake to the [7]. It is traditionally used as bitter, febrifuge and antiscorbutic [8]. It is used as a substitute for *Swertia chirata* for the treatment of fever and jaundice [9]. It is reported that *Limnanthemum indicum* contains different sub types of flavonoids [10]. It is used as a substitute of Ayurvedic drug *Tagara* in the treatment of various diseases like epilepsy [11], anemia, jaundice, tuberculosis [12]. It is reported that the whole plant of *Limnanthemum indicum* is traditionally used as hepatoprotective [13]. *Limnanthemum indicum* has been also reported to show a good anti-proliferative activity [14].

The search for novel natural antioxidants of plant origin has ever since increased. It is not known which constituents of plant are associated in reducing the risk of chronic diseases, but antioxidants appear to play a major role in the protective effect of plant medicine. The present study was designed to investigate the antioxidant activity (in vitro) of ethanolic extract of whole plant of *Limnanthemum indicum*. The extract was also subjected to phytochemical screening to determine the presence of alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, phlobatannins, tannins and terpenoids.

MATERIALS AND METHODS

Collection and Authentication

For the study whole plants of *Limnanthemum indicum* were collected from the local market, Tirunelveli town, Tamilnadu state. It is identified and authenticated by Prof. V.Chelladurai, Botanist, Central council for research in Ayurveda and Siddha, Govt of india, Tirunelveli, Tamilnadu and a voucher specimen was also deposited for future reference.

Extraction

Approximately three kilograms of the whole plant of *Limnanthemum indicum* was collected and washed in running tap water. Then they were chopped into small pieces and dried under shade for about four weeks and finely ground to coarse power in a blender. Initially 150gms of material was packed into the thimble and 2.5 liters of solvent used for extraction was poured into flask (Round Bottom flask). The soxhlet extraction was performed for 18-24 hours until the siphon tube collected solvent appears to be clear. Later the extracted solvent was evaporated under reduced pressure to get dried extract.

Chemicals

Folin-Ciocalteu Reagent, Sodium carbonate, Vanillin reagent, 70% H_2SO_4, Phloroglucinol, Sodium nitroprusside solution, Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%): sulphanilic acid , 20% Glacial acetic, 2, 2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid) diammonium salt) (ABTS) radical cation,

Potassium persulphate, Egg lecithin, Ferric chloride, Ascorbic acid , DMSO, 15% TCA, 0.37% TBA, 5%w/v Carboxy methyl cellulose, Rutin. Phloroglucinol.

Preliminary Phytochemical Studies

The ethanolic extract of whole plant of *Limnanthemum indicum* was tested for different phytoconsituents like alkaloids, glycosides, saponinins, tannins, terpinoids, phenolic compounds, protein, carbohydrates using standard procedures [15].

In-vitro Antioxidant activity

Scavenging of Nitric Oxide radical

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modified Griess Ilosvay reaction [16]. In the present investigation, Griess Ilosvay reagent is modified by using Naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed was measured at 540 nm.The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and extract in DMSO at various concentrations or standard was incubated at 25^{o} C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink coloured chromophore was formed. The absorbance of these solutions was measured at 546 nm and percentage of scavenging activity was measured with reference to ascorbic acid as standard. The nitric oxide radicals scavenging activity was calculated. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples using the equation. IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm.

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Scavenging of 2, 2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation Assay

ABTS assay is relatively recent one, which involves a more drastic radical, chemically produced and, is often used for screening complex antioxidant mixture such as plant extracts, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS radical for the estimation of the antioxidant activity [17,18]. ABTS (54.8 mg) was dissolved in 50 ml of distilled water to 2 mM concentration and potassium persulphate (17 mM, 0.3 ml) was added. The reaction mixture was left to stand at room temperature overnight in dark before use. To 0.2 ml of various concentrations of the extracts or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution was added to make a final volume of 1.36 ml. Absorbance was measured spectrophotometrically, after 20 min at 734 nm.

Lipid peroxidation inhibitory activity

Lipid peroxidation can be initiated by ROS such as hydroxyl radicals by extracting a hydrogen atom from lipids and forming a conjugated lipid radical [19]. This reacts rapidly with oxygen to form a lipid radical until the chain reaction is terminated. The lipid peroxidation adducts may induce the oxidation of biomolecules such as DNA, proteins and other lipids resulting in cellular damage.

The reaction mixture containing egg lecithin (1ml), ferric chloride (0.02ml), ascorbic acid (0.02ml) and extract or standard (0.1ml) in DMSO at various concentrations was kept for incubation for 1 hour at 37ºC. After incubation 2 ml of 15% TCA and 2ml of 0.37% TBA were added. Then the reaction mixture

was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm.

Estimation of Total Phenol content

Total phenolic content (TPC) were determined using Folin-Ciocalteu reagent [20]. Briefly, an aliquot of the sample extract (0.1 ml of 1000 µg/ml in ethanol) was mixed with distilled water (3 ml) and 0.5 ml of Folin-Ciocalteu reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath for exactly 1 min, then cooled and the absorbance was measured at 650 nm using against the reagent blank. The calibration curve was prepared by gallic acid solution (0 - 100µg/ml) in ethanol. TPC was expressed as mg gallic acid equivalent (GAE)/100 g sample dry weight. In a test tube, 200 µl of the extract (1 mg/ml) was mixed with 1 ml of Folin-Ciocalteu reagent and 800 µl of sodium carbonate. After shaking, it was kept for 2 h for reaction. The absorbance was measured at 750 nm. The total phenol content of the extract was determined and expressed as gallic acid equivalent in mg/g of the extract.

Estimation of flavonoid content using Swain and Hillis method (1959)

Aluminum chloride colorimetric method was used to determine Total Flavonoids contents in extracts. [21,22] briefly, an aliquot of 0.5 ml of 2% AlCl3 was added to 0.5 ml of sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm at the final concentration of 1000 µ/ml). TFC was calculated as mg quercetin equivalent (QE) /100 g sample dry weight. The calibration curve was prepared by quercetin solution (0 - 100µg/ml) in ethanol. 0.2 ml of the extract was taken in a test tube and the final volume was made up to 2 ml with distilled water and to this 4 ml of vanillin reagent was added rapidly. Exactly after 15 min. absorbance was recorded at 510 nm against blank. The total flavonoid content of the extract was determined and expressed as pholoroglycinol acid equivalent in mg/g of the extract.

Statistical analysis

Data are expressed as mean ± SD from three separate observations. For in vitro antioxidant assays one way ANOVA test followed by Tukey's test ($P < 0.05$) was used to analyze the differences among IC_{50} of various fractions for different antioxidant assays. The EC_{50} values were determined using the Graph Pad Prism 5 software.

RESULTS AND DISCUSSSION

Preliminary Phytochemical Studies

The ethanolic extract of *Limnanthemum indicum* was screened for the presence of various bioactive compounds of pharmacological importantace using standard methods and it demonstrated the presence of alkaloids, glycosides, saponins, flavanoids, tannins, phytosterols and triterpenoids.

In vitro antioxidant activity

Free radicals and other reactive oxygen species are known to play a definite role in a wide variety of pathological manifestations. The broad range effects produced by them on biological systems have drawn the attention of many experimental works. It has been proven that these mechanisms may be important in the pathogenesis of certain diseases and aging. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms [23].

Nitric oxide radical scavenging activity

Nitric Oxide (NO), a reactive free radical generated from L-arginine by NO synthase, is well documented as a physiological messenger molecule. Excessive amounts of NO, however, are potentially toxic and have been implicated in numerous pathological situations and chronic inflammation [24]. Nitric oxide scavenging activity of ethanolic extract of whole of *Limnanthemum indicum* was detected and compared with Ascorbic acid. The percentage inhibition (% inhibition) at various concentrations (12.5-1000µg/ml) of extract as

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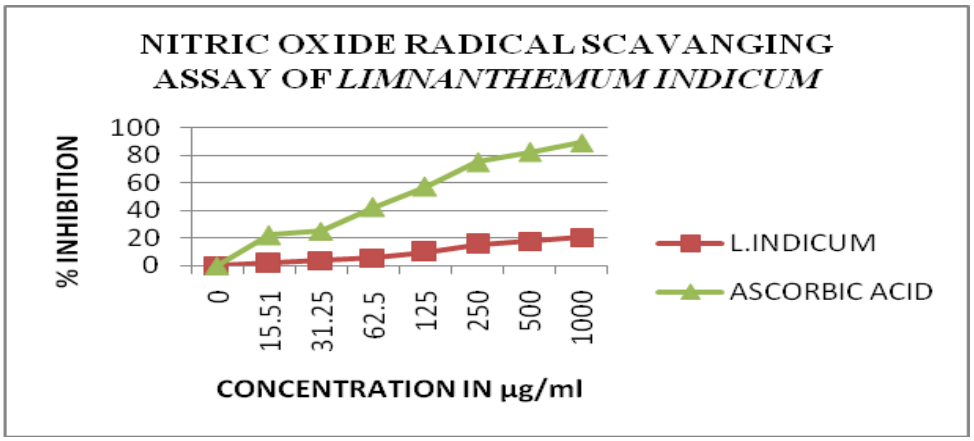
well as Ascorbic acid (10-100µg/ml) were calculated and plotted in Fig.(1) using Microsoft Excel 2007. The IC_{50} values are calculated from graph and were found Ascorbic acid (46.59µg/ml) and ethanolic extract of *Limnanthemum indicum* more than 1000 µg/ml.

Figure 1: Nitric oxide radical scavenging activity of ethanolic extract of *Limnanthemum indicum*

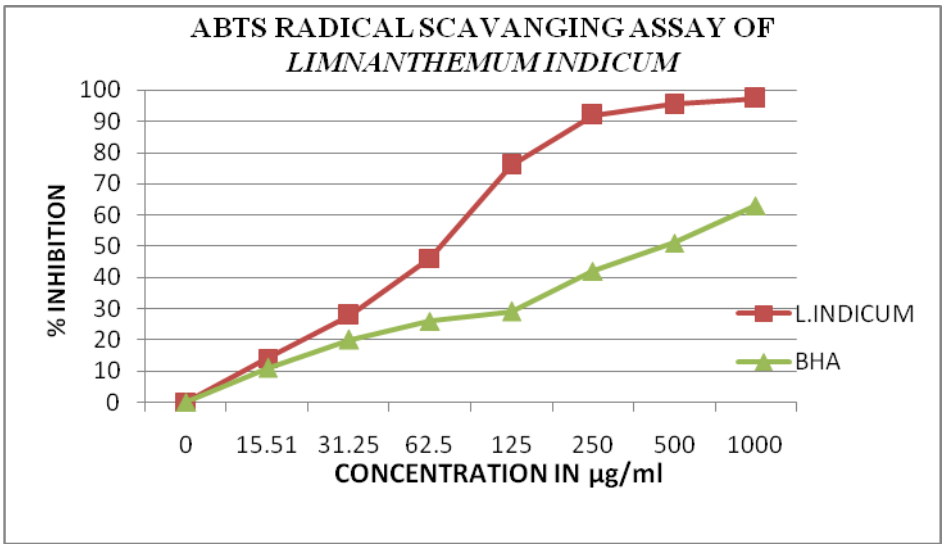


ABTS radical scavenging activity

The antioxidant activity of ethanolic extract of *Limnanthemum indicum* was measured by the method of ABTS radical cation decolourisation assay[25]**.** The improved technique for the generation of ABTS+ described here involves the direct production of the blue/green ABTS+ chromophore through the reaction between ABTS+ and potassium persulphate. Addition of *Limnanthemum indicum* competes with ABTS+ diminishes the color formation.

The antioxidant activity of *Limnanthemum indicum* was assessed by measuring the reduction of the ABTS radical cation as the percentage of inhibition at 734 nm. The effect of various concentrations of extract (15.5-1000µg/ml) on ABTSf+ radical is shown in Fig.(2). *Limnanthemum indicum* exhibited effective antioxidant activity. The inhibition was found to be concentration dependent and the antioxidant activity was comparable with the standard BHA.

Figure 2: ABTS radical scavenging activity of ethanolic extract of *Limnanthemum indicum*



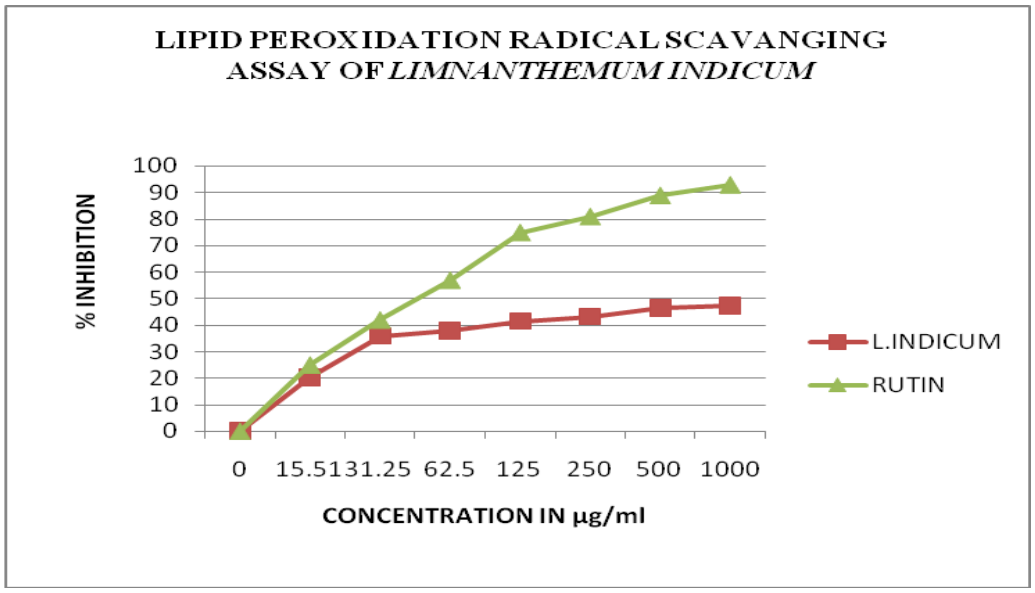
Lipid peroxidation radical scavenging assay

Initiation of the lipid peroxidation by ferrous sulphate takes place either through the ferryl-perferryl complex or through .OH radical by Fenton's reaction. Thus the decrease in the MDA level in egg

phosphatidylcholine with the increase in the concentration of the extract indicates the role of the extract as an antioxidant [26].

The antioxidant activity of *Limnanthemum indicum* was assessed by measuring the reduction of the OH radical cation as the percentage of inhibition at 532 nm. The effect of various concentrations of extract (15.5-1000µg/ml) on lipid peroxidation radical is shown in Fig.(3). *Limnanthemum indicum* exhibited effective antioxidant activity. The IC_{50} values are calculated from graph and were compared with standard Rutin.

Figure 3: Lipid peroxidation radical scavenging activity of ethanolic extract of *Limnanthemum indicum*



Total phenol content

Phenolic compounds are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals [27]. The total phenolic content of ethanolic extract of *limnanthemum indicum* was estimated using Folin–Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent. The phenolic content of the extract was 55.44 mg gallic acid equivalents.

Total flavanoid content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo-γ-pyrone structure. Total flavonoid contents can be determined in the sample extract by reaction with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm [28]. The total flavonoid content was expressed as Phloroglycinol equivalents (RE) in milligram per gram of extract. Total flavonoid content of ethanolic extract of *limnanthemum indicum* was found to be 2.47mg Phloroglycinol equivalents.

CONCLUSION

The results of in vitro antioxidant tests suggested that the ethanolic extract of *limnanthemum indicum* possesses mild free radical scavenging activity which can be attributed to presence of lower concentration of total phenols and flavanoids. However, this plant needs to be evaluated for its antioxidant activity with other solvent extracts and further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract.

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