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Research Article

Evaluation of *In-vitro* Antioxidant Activity and Phytochemical Constituents of Kulekhara (*Hygrophilia spinosa*)

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ABSTRACT

Objectives: This study aimed to evaluate the active phytoconstituents and *in vitro* anti-oxidant as well as free radical scavenging activity of aqueous leaf extract of *Hygrophilia spinosa*, commonly known as kulekhara. Methodology: Phytochemical constituents were measured both by qualitatively and quantitatively using spectrophotometric assays. Total anti-oxidant capacity by phosphomolybdenum reduction, ferric reducing power, cupric ion reducing power and ferrous chelation assays were measured for determination of anti-oxidant activity. *In vitro* DPPH radical, superoxide radical, hydrogen peroxide, hydroxyl radical and nitric oxide scavenging activities were also estimated using spectrophotometry. Result: Our study showed that the leaf extract of *H. spinosa* contains active phytoconstituents like polyphenols, tannins and alkaloids. Furthermore, it showed cupric as well as ferric ion reducing capability and selective scavenging ability towards free radicals such as superoxide anion and hydrogen peroxide. Conclusion**:** This study elucidated that leaf extract of *H. spinosa* possesses concentration dependent superoxide and hydrogen peroxide scavenging activity. These results suggested the potentials of *H. spinosa* as a preventive medicine against free radical associated oxidative assault.

INTRODUCTION

Medicinal plants are of great importance to health of individuals and communities. The role of medicinal plants in disease prevention or control has been attributed largely to antimicrobial, anti-diabetic, hypolipidemic, anti-carcinogenic, anti-inflammatory as well as anti-oxidant activity. The plant products that are derived mainly from leaves, fruits, seeds, root or even stem barks are known to act as phytomedicine and influence various physiological functions in human body. All these plants products contain one or more natural bioactive substances like alkaloids, tannins, phenolic compounds, flavonoids and saponin. Among these substances the anti-oxidant property is largely associated with amphipathic compounds like polyphenols[1] and in recent years reports of anti-oxidant property of plant products has been increased immensely[2].

Reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) as also nitric oxide ($NO^{\cdot}$), peroxynitrite anion ($ONOO^-$) play an important role in oxidative stress and are related to pathogenesis of important diseases like cancer, Alzheimer's, diabetes, arthritis etc[3]. ROS induced damage primarily targets biological macromolecules and causes reaction such as lipid peroxidation, protein carbonylation, protein nitrosylation, oxidative damage to DNA etc[3]. In healthy individuals, there is a stoichiometric balance of free radicals and anti-oxidant systems (both enzymatic and non-enzymatic); however, oxidative stress is generated when the equilibrium is tilted towards free radicals either due to increased generation of free radicals or depletion of anti-oxidant levels[4]. Anti-oxidant molecules act either by inhibiting the process of free radical generation or can directly scavenge the ROS or chelate the transition metals that catalyze the free radical generation[5]. To overcome the oxidative stress, in recent years, synthetic anti-oxidants have been developed like gallic acid esters, tertiary butylated hydroquinone but they are suspected to have some adverse effects on health. Hence, their use are restricted and replaced with naturally occurring anti-oxidants as the later are safe, having minimum side effects compared to the synthetic ones[6,7].

Hygrophilia spinosa, commonly known as kulekhara belonging to the family Acanthaceae and has been used in Ayurvedic literature from ancient times[8]. This plant is commonly found in India, Srilanka, Myanmar and Nepal. Importantly, various part of this plant has been used for controlling diseases including anemia, leukorrhea, gonorrhoea, asthma, blood diseases, hyperdipsia, dysentery, cancer, gastric diseases, and inflammation etc[8]. Phytochemically, the whole plant contains almost all bioactive substances such as tannins, phytosterols, alkaloids, polyphenols, carbohydrates etc[8] which entails for its multiple activities.

The present study was carried out to explore the *in vitro* anti-oxidant activity and phytoconstituents of the leaf extract of *Hygrophilia spinosa*.

MATERIALS AND METHODS

Chemicals

**Author for Correspondence*

Figure 1

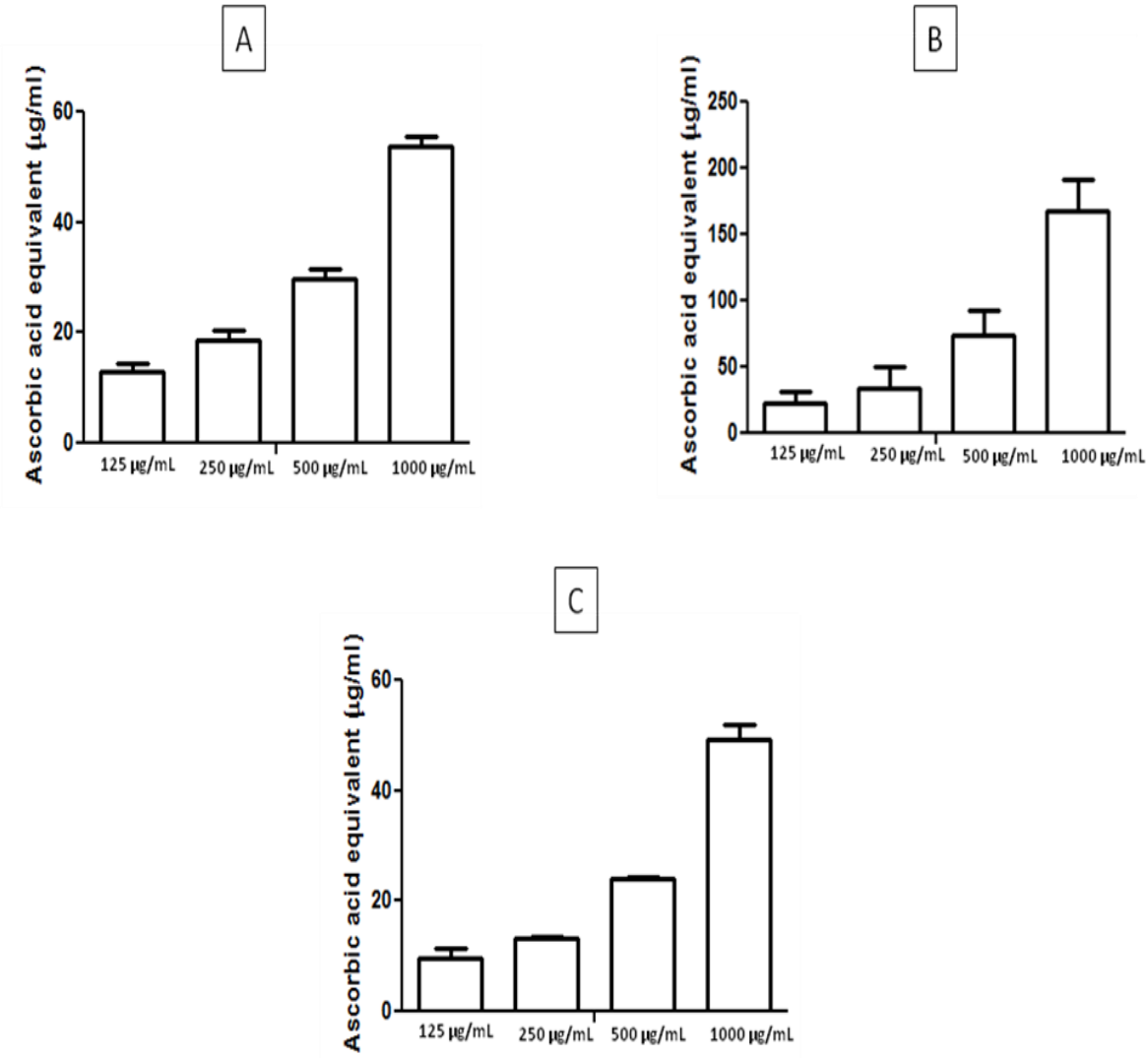

Figure 1: Anti-oxidant activity of *H. spinosa* leaf extract

A**.** Bar diagram showing total anti-oxidant capacity of *H. spinosa* leaf extract. X axis indicates concentration of the leaf extract and Y axis is plotted against concentration of ascorbic acid.

B. Bar diagram showing ferric reducing anti-oxidant power (FRAP) of *H. spinosa* leaf extract. X axis indicates concentration of the leaf extract and Y axis is plotted against concentration of ascorbic acid.

C. Bar diagram showing cupric ion reducing power (CUPRAC) of *H. spinosa* leaf extract. X axis indicates concentration of the leaf extract and Y axis is plotted against concentration of ascorbic acid.

All the chemicals were purchased from Loba Chemie (Mumbai, India), Sisco Research Laboratory (SRL, Mumbai, India), and Merck (Bangalore, India) or from HiMedia (Mumbai, India). All the chemicals purchased were of analytical grade.

Preparation of sample

The plant material was purchased from Alberta Agro Pvt. Ltd, Kolkata. The leaf powder of *H. Spinosa* (500 mg) was mixed with 50 mL of Milli-Q grade water, stirred well and then filtered with Whatman filter paper grade 1 to make a stock concentration of 10 mg/mL. The working stock of 1 mg/mL was prepared fresh just before experiment. The stock was stored at 4^0C.

Phytochemical analysis

Qualitative tests

Phytochemical analysis of water extract of *H. Spinosa* was carried out using standard qualitative methods as described previously[9,10]. The components analyzed for phytochemicals were alkaloids, tannins, phenols, Phlobatannin, saponin, flavonoids, sterols, essential oil, carbohydrate, reducing sugars, amino acids, terpenoids, diterpenes and proteins.

Quantification of total phenolic contents

Total phenolic content was determined using the Folin-Ciocalteu (FC) reagent as per ISO[11]. Briefly, 1 mL of plant extract (1 mg/mL) was mixed with 5 mL of FC reagent, mixed and incubated for 8 minutes. After that 4 mL of sodium carbonate solution (7.5% w/v) was added

Figure 2

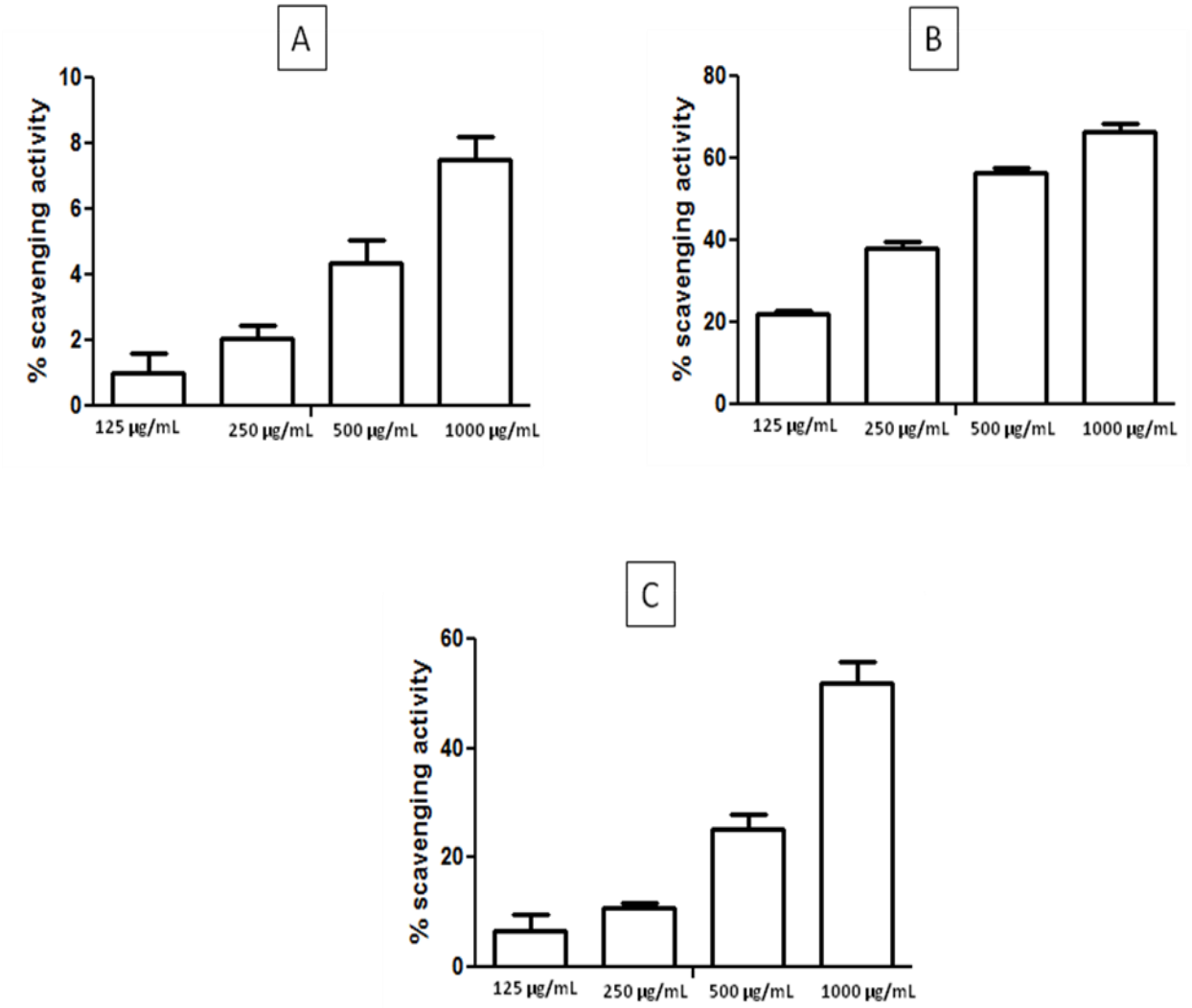

Figure 2: Free radical scavenging activity of *H. spinosa* leaf extract
A. Bar diagram showing DPPH radical scavenging capacity of *H. spinosa* leaf extract. X axis indicates concentration of the leaf extract and Y axis means percentages of scavenging activity.
B. Bar diagram showing superoxide radical scavenging capacity of *H. spinosa* leaf extract. X axis indicates concentration of the leaf extract and Y axis means percentages of scavenging activity.
C. Bar diagram showing hydrogen peroxide scavenging capacity of *H. spinosa* leaf extract. X axis indicates concentration of the leaf extract and Y axis means percentages of scavenging activity.

and mixed. The solution was then allowed to stand for 60 minutes and optical density was measured at 765 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against appropriate sample blank and reagent blank. The concentration of phenolic content was determined against a gallic acid standard curve (0 – 50 µg/mL).

Quantification of tannin content

This was performed by vanillin hydrochloride method described by Robert[12] with slight modifications. A volume of 0.4 mL of extract (1 mg/mL) was added to 3 mL of a solution of vanillin (4% w/v in methanol) and 1.5 mL of concentrated hydrochloric acid. After 20 minutes of incubation, the absorbance was read at 500 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). The concentration of tannin was determined against a tannic acid standard curve (0 -100 mg/mL).

Quantification of reducing sugar content

Quantitative estimation of reducing sugar was estimated using 3,5-dinitro salicylic acid method. Briefly, 1.5 mL of extract (1 mg/mL) was mixed with 1.5 mL of water and 3 mL of 3,5 dinitro salicylic acid reagent (5% w/v dinitro salicylic acid in 2M NaOH mixed with 60% sodium potassium tartarate), mixed well and incubated in a boiling water bath for 15 minutes. Finally, absorbance was recorded at 520 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). Concentration of reducing sugar in the plant extract was

Table 1: Qualitative test of phytochemicals in Kulekhara extract

Parameter	Test	Result
Tannin	Prussian blue test	++
Phlobatannin	Hydrochloric test	-
Saponin	Foam formation test	+
Flavonoids	Alkaline reagent test/Lead acetate test	-
Terpenoids	Salkowski test	++
Phenols	Ferric chloride test	+
Sterol	Salkowski test	-
Essential oil	Acid-alkali test	-
Alkaloids	Potassium dichromate test	++
Carbohydrates	Molish's test	++
Reducing sugars	Fehling's test	++
Amino acids	Ninhydrin test	-
Protein	Biuret test	-
Diterpenes	Copper acetate test	-

calculated against a standard curve of dextrose (0 -1000 µg/mL).

In vitro anti-oxidant and free radical scavenging activity

Total anti-oxidant capacity

The total antioxidant capacity of the extract was evaluated by the phosphomolybdenum method according to the procedure described earileir[13]. Briefly, 0.2 mL of different concentrations of extract was combined with 1.8 mL of water and 2 mL of reagent solution (0.31 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^0C for 90 min. Then, the absorbance of the solutions were measured at 695 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific) against blank after cooling to room temperature. Water (0.2 mL) in the place of extract was used as the blank. The total antioxidant activity is expressed as the µg /mL equivalent of ascorbic acid (AAE). The calibration curve was prepared by mixing ascorbic (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) with water.

Ferric ion reducing anti-oxidant power assay (FRAP)

The ferric ion reducing ability was determined by the method described previously[14] with some modifications. Briefly, 0.04 mL of different concentrations of plant extract was added to 2 mL of deionized water and 0.36 mL of 95% ethanol. To this, 0.6 mL of 1M HCl and 0.6 mL of 1% (w/v) potassium ferricyanide were added. Then 0.2 mL of 1% (w/v) sodium dodecyl sulphate and 0.4 mL of 0.2% (w/v) ferric chloride were added to the solutions and mixed well by vortexing. Tubes were then placed to floter and kept in 50^0 C in water bath for 20 minutes. After incubation in water bath, tubes were taken out and allowed to rapid cooling. Reading of absorbance was taken at 700 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). The ferric reducing power is expressed as the µg/mL equivalent of ascorbic acid (AAE). The calibration curve was prepared by mixing ascorbic (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) with water.

Cupric ion reducing power (CUPRAC)

The cupric ion reducing power was determined using the method described elsewhere[15]. Briefly, 0.6 mL each of double distilled water, cupric chloride (0.01 M), sodium citrate (25% w/v) were added to tubes respectively and mixed thoroughly. Then different concentrations of plant extract was added to tubes and again mixed thoroughly. Finally, 0.6 mL neocuproine (0.0075M) was added. The mixture then was mixed well by vortexing and tubes were incubated at room temperature for 10 minutes. After incubation, reading of absorbance was taken at 450 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). The cupric reducing power is expressed as the µg/mL AAE. The calibration curve was prepared by mixing ascorbic (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) with water.

Ferrous ion chelating assay

The chelating effect on ferrous ions of the prepared extracts was estimated by the method of Dinis with slight modifications[16]. Briefly, 3 mL of test sample (different concentrations of *H. Spinosa* extract) was taken and added to 0.15 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.3 mL of 5 mM ferrozine into the mixture, which was then kept at room temperature for 10 minutes and then the absorbance of the mixture was determined at 562 nm. Ethylene di amino tetra acetate (EDTA) was used as standard reference compound. The % of ferrous ion chelating ability was calculated by the following formula-

Fe^{+2} chelating ability in % = [1-(test sample absorbance/blank sample absorbance)] X 100%

DPPH radical scavenging assay

DPPH (1,1, Diphenyl-2-Picrylhydrazyl) scavenging activity was carried out by the method described earlier[17] with some modifications. 0.15 mL of 3.3 mM DPPH were added to 3 mL of absolute ethanol in multiple tubes and mixed thoroughly. Different concentrations of plant extract were then added to the tubes. The mixture then was mixed well by vortexing for 10-15 seconds. Tubes were then incubated at room temperature for 30 minutes in dark at room temperature. After incubation, reading of absorbance was recorded at 517 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). Ascorbic acid was used as positive control for the experiment. The % of DPPH radical scavenging activity was calculated using the following formula-

$$\% \text{ of scavenging activity} = \frac{\text{Absorbance of control- Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging ability was determined by a new method using 1,10-phenanthroline. Briefly, 0.25 mL of ferrous ammonium sulphate (1 mM), added to the test-tube and then 1.5 mL of different concentrations of plant extract were added and mixed. After that 62.5 µL of 5 mM hydrogen peroxide was added to each tube and mixed, incubated for 5 minutes at room temperature, in dark. Following this, 1.5 mL of 1,10-phenanthroline monohydrate was added to the tubes and incubated for 10 minutes in dark at room temperature. After incubation,

Table 2: Quantitative test of phytochemicals in Kulekhara at 1 mg/mL concentration

Parameter	Test	Result
Tannin	Prussian blue test	1.855 ± 1.87 mg/mL
Phenols	Folin-Ciocalteu test	21.83 ± 2.78 µg/mL
Reducing sugars	3,5 Dinitro salicylic test	173.92 ± 2.53 µg/mL

absorbance was read at 510 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). Ascorbic acid was used as positive control. The % of hydrogen peroxide scavenging activity was calculated using the following formula-

% of scavenging activity =

$$\frac{\text{Absorbance of blank- Absorbance of test}}{\text{Absorbance of blank}} \times 100$$

Superoxide radical scavenging assay

Superoxide radical scavenging assay was performed as described by Babu et al.[18] with some modification using NADH-PMS system. 1 mL of NBT solution (0.156 mM), 1 mL of NADH solution (0.468 mM) and 1 mL of plant extract of different concentration were added and mixed. The reaction was started by addition of 1 mL of PMS (0.06 mM) to the mixture. The reaction mixtures were incubated at room temperature for 5 minutes in dark. After incubation, reading of absorbance was taken at 560 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). Ascorbic acid was used as positive control. The percentage of superoxide radical scavenging activity was calculated by the following formula-

% of scavenging activity =

$$\frac{\text{Absorbance of control- Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Hydroxyl radical scavenging assay

This assay was performed as described by Kumar et al.[14] based on the degradation of deoxy ribose sugar and tested for malonaldehyde formation. To the tubes 1.5 mL of phosphate buffer, 0.5 mL of 10 mM 2-Deoxy ribose, 0.25 mL of 20 mM $FeCl_2$ solution, 0.25 mL of 20 mM EDTA solution, 0.1 mL of different concentration of plant extract were added, 1.9 mL distilled water and 0.5 mL of 10 mM hydrogen peroxide were added rapidly following the order. The mixtures having a total volume of 5 mL were incubated for 4 hrs at 37^0 C in a water bath. At the end of period, the reaction is arrested by adding 2.5 mL of 2.8% tri chloro acetic acid (TCA). To this 2.5 mL of 1% thiobarbituric acid (TBA) was added, kept the reaction mixture at 100^0 C in a boiling water bath for 10 minutes. The mixture was allowed to cool under running tap water and read the absorbance read at 532 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). Mannitol was used as positive control. The percentage of hydroxyl radical scavenging activity was calculated by the following formula

[1-(test sample absorbance/blank sample absorbance)] X 100 %

Nitric oxide scavenging activity

Nitric oxide scavenging potential was determined by using the Griess reaction where sodium nitroprusside acts as nitrite donors[19] 1 mL of plant extract in different concentration and 1 mL of 10 mM Sodium Nitroprusside (in 10 mM phosphate buffer) were mixed in the test tubes and mixed thoroughly. Then the mixture was incubated for 3 hours at room temperature (25^0C). After incubation 1 mL of griess reagent A (1% w/v sulphanilamide in 5% phosphoric acid) added to the mixture, mixed and incubated for 20 minutes at 30^0C. Finally, 1 mL of griess reagent B (0.1% w/v naphthylethylenedimaine Dihydrochloride or NED in water) was added to the mixture, mixed and incubated for 10 minutes in dark at 30^0C. After incubation, reading of absorbance then should be taken at 546 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Scientific). Ascorbic acid at a concentration of 125 µg/mL was used as positive control. The percentage of nitric oxide scavenging activity was calculated by the following formula-

% of scavenging activity =

$$\frac{\text{Absorbance of control- Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Statistical analysis

All the assays were repeated three times (n = 3). Values were expressed as mean ± standard deviation (SD). Statistical analysis was done using GraphPad prism version 5.0 software (Graph Pad Software Inc., La Jolla, CA, USA).

RESULTS

Preliminary phytochemical screening of the aqueous extract of leaves of *H. spinosa* revealed that it contains tannins, saponin, terpenoids, phenolics, alkaloids and reducing sugars [Table 1]. The quantitative phytochemical estimation indicated that it contains significant amount of reducing sugars, tannins and other phenolics [Table 2]. Importantly, according to ayurveda, siddha, folk and Chinese traditional recipe tannins, phenolics and alkaloids are the important ingredients to prevent oxidative stress[14]. Our study revealed that leaf extract of *H. spinosa* contains these bioactive substances and could be developed as a valuable source of anti-oxidant.

Total anti-oxidant activity

Phosphomolybdenum assay used to determine the total anti-oxidant capacity was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green coloured phosphate/Mo (V) complex at acidic pH[13]. Our study showed that leaf extract of *H. spinosa* showed an increased total anti-oxidant capacity in a dose dependent manner starting at 125 µg/mL [12.88 ± 2.30 AAE, Figure 1A] and found to be optimum at 1000 µg/mL [53.86 ± 2.30 AAE, Figure 1A]. The obtained ascorbic acid equivalent (AAE) value demonstrated that the extract is a moderate anti-oxidant.

Ferric reducing power

Anti-oxidants are also reducing agents by donating hydrogen atom. The ferric ion reducing ability of the

extract was evaluated in comparison with ascorbic acid. Study revealed that the leaf extract was able to reduce ferric ion in a dose dependent manner ranging from 125 µg/mL [22.63 ± 12.98 AAE, Figure 1B] to 1000 µg/mL [167.68 ± 33.76 AAE, Figure 1B].

Cupric reducing power

The CUPRAC assay utilizes copper (II)-neocuproine {Cu (II)-Nc} reagent as the chromogenic oxidizing agent. It is based on the measurement of absorbance at 450 nm by the formation of stable complex between neocuproine and copper (I)[15]. The cupric ion (Cu^{2+}) reducing ability of aqueous leaf extract of *H. spinosa* is shown in Figure 1C. Importantly, Cu^{2+} reducing capability measured by this method was found to be concentration-dependent akin to total anti-oxidant activity and ferric reducing ability. However, in comparison to ferric ion reducing property the ability to reduce cupric ion was found to be 3.4 fold lowered as in 1000 µg/mL it gave 49.34 ± 3.70 AAE [Figure 1C] whereas in FRAP it gave 167.68 ± 33.76 AAE.

Ferrous ion chelation

The chelating of Fe^{2+} by extracts was estimated by the method Decker and Welch[16]. Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted resulting in decrease in the red colour of the complex. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the plant extracts. The ability of chelating Fe^{2+} is considered as significant because transition metal ion like Fe^{2+} possess the ability to move single electrons thus allowing the formation and propagation of many radical reactions[20]. However, our study showed that the kulekhara leaf extract (*H. spinosa*) was unable to chelate Fe^{2+} whereas the reference compound EDTA, a known chelator, was able to chelate Fe^{2+} completely in a dose dependent manner. Hence the data is not shown here.

DPPH radical scavenging activity

We found that the extract was able to scavenge only 1% - 8% of DDPH radical [Figure 1D] which is very unconvincing. However, as DPPH is not a physiologically relevant radical, this finding warranted further study with physiologically relevant ROS such as superoxide radical, hydrogen peroxide etc.

Superoxide anion avenging activity

Superoxide anion is a very potent ROS that damages many biomolecules like proteins, nucleic acids. Here, in this study, the PMS-NADH coupling reaction yields superoxide radicals that is measured by its ability to reduce the NBT to a purple formazan. The aqueous leaf extract of *H. spinosa* showed very good superoxide scavenging activity as shown in Figure 1E. The extract showed a dose dependent increase in superoxide radical scavenging activity ranging from 125 µg/mL [scavenging activity = 17.67 ± 7.34 %, Figure 1E] to 1000 µg/mL [scavenging activity = 63.44 ± 7.07 %, Figure 1E].

Hydrogen peroxide scavenging activity

In physiological condition, superoxide radical can be decomposed to generate hydrogen peroxide, which although itself is a weak oxidizing agent, but further can generate hydroxyl radical by reacting with ferrous ion. Our study showed that the *H. spinosa* leaf extract possess substantial hydrogen peroxide scavenging activity ranging from 6.65 ± 4.29 % (at 125 µg/mL, Figure 1F) to 52.07 ± 5.20 % (at 1000 µg/Ml, Figure 1F).

Hydroxyl radical scavenging activity

Hydroxyl radical is also a very potent toxic radical that is harmful to almost every biomolecules. We have tested our extract for its ability of hydroxyl radical scavenging activity, but the extract was found to be unable to scavenge hydroxyl radical and hence not shown.

Nitric oxide scavenging activity

Apart from common ROS, nitric oxide is also known for its diverse action in immunity and physiology. The production of nitric oxide radicals can result in inflammation, vasodilatation and even septic shock. The toxicity increases greatly when it forms the highly reactive peroxynitrite anion ($ONOO^-$). The present study showed that the kulekhara leaf extract (*H. spinosa*) was unable to scavenge nitric oxide and hence the data was not presented.

CONCLUSION

In this study, it was determined that the aqueous leaf extract of H. spinosa has considerable anti-oxidant and metal ion reducing activity however, with regard to free radical scavenging action, it was selective for superoxide and hydrogen peroxide. Furthermore, phytochemical constituents revealed that it contains tannins and polyphenols which can possibly account for its free radical scavenging action. One plausible explanation for its selective action on free radicals could be the lack of presence of flavonoids in it which is known to be a good scavenger of nitric oxide and hydroxyl radicals[21]. This research proved that kulekhara (*H. spinosa*) could be an easily accessible source of good anti-oxidants but further studies are required to establish its action *in vivo*.

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