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Research Article

EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITY OF INDIAN MEDICINAL PLANT *HYDNOCARPUS PENTANDRA*

SHYAM KRISHNAN. M[1], DHANALAKSHMI. P[2], YAMINI SUDHALAKSHMI. G[3], GOPALAKRISHNAN. S[4], MANIMARAN. A[5], SINDHU. S[6], SAGADEVAN. E[6*] AND ARUMUGAM. P[6]

[1]Department of Biochemistry, SRM Medical College Hospital & Research Centre, Kattankulattur 603203 Tamilnadu, India, [2]Biocontrol & Microbial Metabolites Lab, CAS in Botany, University of Madras, Maraimalai Campus, Guindy, Chennai 600025, [3]Dept. of Biochemistry, Professor Dhanapalan College of Arts and Science, Kelambakkam, Chennai 603103, [4]Dept. of Applied Sciences, PNG University of Technology, Papua New Guinea, [5]Dept. of Biological sciences, Aarupadai Veedu Institute of Technology, Paiyanoor, Chennai 603104, [6]Armats Bioproducts Unit, ARMATS Biotek Private Limited, Maduvankarai, Guindy, Chennai 600032, Tamilnadu, India. Email: gingeesaga@gmail.com

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ABSTRACT

Objective: The study was conducted to investigate the phytochemical profile and the total antioxidant and free radical scavenging activities of Indian Medicinal Plant *Hydnocarpus pentandra*.

Methods: Two different solvents such as ethyl acetate and methanol were used to extract the crude bio-active compounds from whole *H. pentandra* plant. The total antioxidant and free radical scavenging activities of *H. pentandra* extracts were measured by ferric thiocyanate (FTC), thiobarbituric acid (TBA) and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) methods.

Results: Qualitative phytochemical analysis showed phenolics, carbohydrates, saponins and glycosides were in high amounts. In the bioautography, it was observed that the whole plant extract had free radical scavenging activity and thus a broad spectrum was obtained at the R_f of 0.77. In case of radical scavenging activity, methanolic extracts showed 44% efficiency and 66% for ethyl acetate which was adequately comparable to the radical scavenging activity of the standard α-tocopherol (84%). The Super Oxide Dismutase assay was also conducted for both the extracts and the ethyl acetate extracts (0.402) exhibited highest activity among the extracts which is closer to the activity of the α-tocopherol standard (0.632).

Conclusion: Overall results concluded that the *Hydnocarpus pentandra* plant is a potent source of natural antioxidants. Further analysis on the mechanism of these antioxidants and other beneficial compounds from various plants are needed for a better understanding to implement them into functional foods.

Keywords: Antioxidants, Radical scavengers, Medicinal plants, *Hydnocarpus pentandra,* Phytochemicals

INTRODUCTION

Medicinal plants based traditional systems of medicines are playing important role in providing health care to large section of population, especially in developing countries. Interest in them and utilization of herbal products produced based on them is increasing in developed countries also [1]. India has the unique distinction of having six recognized systems of medicine in this category. They are- Ayurveda, Siddha, Unani and Yoga, Naturopathy and Homoeopathy. Though Homoeopathy came to India in 18th Century, it completely assimilated in to the Indian culture and got enriched like any other traditional system hence it is considered as part of Indian Systems of Medicine [2]. All the above systems of medicine in India are largely based on herbal drugs. Despite of their efficient effects, much of the ancient knowledge and many valuable medicinal plants of India are being lost at an alarming rate. With the rapid depletion of forests, impairing the availability of raw drugs, Ayurveda, like other systems of herbal medicines has reached a very critical phase. In this context, scientific validation of Indian medicinal plants is the need of hour. The medicinal value of plants can be attributed to some chemical substances which produce a definite physiological action on the human body. The most important of such bioactive compounds are alkaloids, flavonoids, tannins and phenolic compounds [3].

Hydnocarpus pentandra (Buch.-Ham.) Oken is a medicinal herb that belongs to the family Flacourtiacea and is available in moist deciduous and semi-evergreen forests of Western Ghats, India up to 850 m. Seeds yield Hydnocarpus or chaulmoogra oil that is used in leprosy. Seed oil is anti-inflammatory, alterative, tonic, used as local application in rheumatism, sprains, braises sciatica and chest affections. Seed and seed oil are also used in leucoderma, worm infection, polyuria, pruritus, eye diseases and sinus wounds. There has been very few scientific studies conducted on *H. pentandra* however most of them have considered this plant one among several plants [4,5]. Especially, Kshirsagar and Upadhyay [5] have studied the free radical scavenging activities of *H. pentandra* along with several other plants. However, a comprehensive and exclusive study of the medicinal properties of *H. pentandra* is still lacking.

Free radicals and reactive oxygen metabolites can react with proteins, nucleic acids and lipids, causing changes in genetic material and inactivation of enzymes. Free radicals have been implicated in the etiology of several degenerative disorders including cancer, diabetes, rheumatoid arthritis, atherosclerosis, liver cirrhosis, Alzheimer's disease and other neurodegenerative disorders [6].Therefore, the human health depends on the efficiency of antioxidant mechanisms. Because of detrimental influence of peroxides and oxygen radicals on organism there is growing interest in natural antioxidants, especially in polyphenols [7]. Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer [8]. Hence, the screening studies for antioxidant properties of medicinal and food plants have been performed increasingly for the last few decades. In this context, Indian medicinal plant H. pentandra is investigated in the present study for phytochemical profile and antioxidant activities. Though this medicinal plant has been subjected to some scientific studies, their phytochemical constituents and comprehensive study on the antioxidant activity is not yet approached comprehensively. Hence, the present study assumes much significance in scientific validation of Indian Medicinal plants.

MATERIALS AND METHODS

Preparation of *H. pentandra* extract

Fresh plant of *Hydnocarpus pentandra* was collected from the natural locations in Aanaimalai region at Pollachi, Tamil Nadu. The plant collected was cleaned with water and dried in room temperature for one week, ground into powder and stored in room temperature. Direct extraction with ethyl acetate and methanol,

following the previously published standard methods [9], was done for the purpose of preliminary screening of the *H. pentandra*. In this method, finely ground material (10 gm) was extracted thrice with 100ml of ethyl acetate and methanol in conical flask under shaking condition. The extract was concentrated by condensation, weighed and re-dissolved in different solvents to yield 10 mg/ml solutions for further analysis.

Analysis of crude extract for preliminary screening

Thin Layer Chromatography

The crude extract (10mg/ml DMSO) was spotted on the TLC plates and dried. It was then run with different solvent in varying ratio. The spots were identified in UV light, far light and in iodine chamber. Also the R_f value was calculated. The compounds from the spots were scraped and used for further screening.

Bioautography

Developed standardized chromatographic plates of crude extract was sprayed with free radical of DPPH (dissolved in Ethanol) and the zone of inhibition was seen immediately after sprayed. The specific compound (band) having antioxidative properties showed clear zone. This method was chosen for its simplicity, low cost, accuracy and rapid results make it ideal for bioassay guided isolation[7].

Phytochemical Screening

The different qualitative chemical tests were performed for establishing the profile of given extract for its chemical composition. The following tests were performed using standard protocols on the extracts to detect various phyto constituents present in them as described in our previous publication [10]. Basic methods were done using the standard methods [18,19].

Detection of alkaloids

Alkaloids were detected by following previously published method[8] in which solvent free extract (50 mg) was stirred with few ml of dilute hydrochloric acid and filtered. To a few ml of filtrate, a drop or two of Mayer's reagent was added by the sides of the test tube. A white creamy precipitate indicates the test as positive.

Detection of carbohydrates

The extract (100mg) was dissolved in 50 ml of distilled water, filtered and subjected to the following tests [11].

Fehling's test

One ml of filtrate was boiled on water bath with 1 mL each of Fehling's solution I and II. A red precipitate indicated the presence of sugar.

Benedict's test

To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added and heated on a boiling water bath for 2 min. a characteristic colored precipitate indicated the presence of sugar.

Detection of glycosides

50 mg of extract was hydrolyzed with concentrated hydrochloric acid for 2h on a water bath, filtered and the hydrolysate was subjected to the following tests as suggested previously [11].

Borntrager's test

To 2 ml of filtrate hydrolysate, 3ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicated the presence of glycosides.

Detection of saponins: Foam test [19]

The extract (50 mg) was diluted with distilled water and made up to 20 ml and the suspension was shaken in a graduated cylinder for 15 min. A 2cm layer of foam indicated the presence of saponins.

Detection of proteins and amino acids [12]

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatman No.1 filter paper and the filtrate was subjected to tests of proteins and amino acids.

Millon's test [13]

To 2 ml of filtrate, few drops of millon's reagent were added. A white precipitate indicated the presence of proteins.

Biuret test

An aliquot of 2 ml of filtrate was treated with one drop of 2% copper sulphate solution. To this, l ml of ethanol (95%) was added, followed by addition of excess of potassium hydroxide pellets. Pink color in the ethanolic layer indicated the presence of proteins.

Detection of Phenolic Compound

Ferric chloride test [14]

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green color indicated the presence of phenolic compounds.

Lead acetate test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, 3ml of 10% lead acetate were added. A bulky white precipitate indicated the presence of phenolic compounds.

Estimation of Radical Scavenging Activity (RSA) Using DPPH Assay [10]

The RSA activity of different extracts was determined using DPPH assay as described previously [10]. The decrease in absorption of the DPPH solution at 517 nm was monitored after 20 min on addition of 40 µl of 20 - 200 µg/mL of plant extract in a cuvette. Ascorbic acid (AA) and Butylated hydroxy toluene (BHT) were used as references. The ability to scavenge DPPH radical was calculated by the following equation.

$$\% \text{ of DPPH radical scavenging activity (\% RSA)} = \frac{Abs_{control} - Abs_{sample} \times 100}{Abs_{control}}$$

$Abs_{control}$ is the absorbance of DPPH radical + ethanol; Abs_{sample} is the absorbance of DPPH radical + leaf extract. Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions. The IC_{50} (Concentration providing 50% inhibition) was calculated graphically using a calibration curve Vs percentage of inhibition.

Ferric thiocyanate (FTC) method [10]

The leaf samples of 4 mg in 99.5% ethanol were mixed with 2.51% linoleic acid in 99.5% ethanol (4.1 mL), 0.05 M phosphate buffer, pH 7 (8 mL) and distilled water (3.9 mL) and kept in screw cap containers under dark conditions at 40°C. To 0.1 mL of this solution, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added. After 3 min, 0.1 mL of 2 M ferrous chloride in 3.5% HCl was added to the reaction mixture and the absorbance of the red color was measured at 500 nm each 24 h until one day after absorbance of the control reached maximum. The control and the standard were subjected to the same procedure as the sample except for the control, where there was no addition of sample, and for the standard 4 mg of sample were replaced with 4 mg of α-tocopherol or BHT.

Thiobarbituric acid (TBA) test [10]

The same samples as prepared for the FTC method were used in TBA test. To 1 mL of sample solution, 2 mL each of 20% aqueous trichloroacetic acid were added. This mixture was then incubated in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and the absorbance of supernatant was measured at 532 nm. Antioxidative activity was recorded based on absorbance on the final day.

Superoxide Anion Radical Scavenging Assay [15]

To 1ml of NBT solution (156µM NBT in 100mM phosphate buffer, pH8), 1ml of NADH solution (468µM in 100mM phosphate buffer, pH8) was added and 0.1ml of sample solution were mixed to it. The reaction was started by adding 100µl of PMS solution (60µM PMS in 10mM, Phosphate buffer, Ph 8). The Mixture is incubated at 25ºc for 5minutes and the absorbance is measured at 560nm.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of ethyl acetate extracts of *H. pentandra* was measured according to the standard method [16]. Various concentrations (50, 100, 150 and 200µg) of extracts were added with 1.0ml of iron-EDTA solution, and 1.0ml of dimethyl sulphoxide (DMSO). The reaction was initiated by adding 0.5ml of ascorbic acid (0.22%) and incubated in water bath at 80–90°C for 15 min. After incubation the reaction was terminated by the addition of 1.0ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3.0ml of glacial acetic acid, and 2ml of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at room temperature for 15min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity is calculated by the following formula:

% HRSA = from [(A0 - A1)/A0] X100, where A0 is the absorbance of the control and A1 is the absorbance of the extract/standard.

Metal chelating activity

The chelating of ferrous ions by ethyl acetate extracts of *H. pentandra* was estimated by the previously published method [17]. Briefly the extract samples (250 µl) were added to a solution of 2 mM/l FeCl2 (0.05ml). The reaction was initiated by the addition of 5 mM/l ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract.

Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al.* An aliquot of 100µl of sample solution was combined with 1ml of reagent solution in a 4ml vial. The vials were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The results reported (Ascorbic acid equivalent antioxidant activity) are mean values expressed as g of ascorbic acid equivalents/100g extract.

RESULTS

Phytochemical screening of ethyl acetate extract of *H. pentandra*

The preliminary phytochemical screening of *H. pentandra* revealed the presence of phenolics, carbohydrates, saponins and glycosides in high amounts, whereas, proteins was present in trace amounts (Table 1).

Table 1: Phytochemical profile of *H. pentandra*

Compound	Result
Alkaloids	-
Carbohydrates	+
Glycosides	++
Saponins	+
Protein	+
Phenol	+

Thin Layer Chromatography

The chromatogram developed with 20% ethyl acetate in hexane revealed the presence of eight major compounds with R_f values of 0.28, 0.35, 0.42, 0.53, 0.64, 0.77, 0.83 and 0.93 as visualized under iodine vapour and UV illumination (Figure 1).

Bioautography

TLC plate run with 20% ethyl acetate in hexane ratio was selected and the plate was sprayed with DPPH (free radical). It was observed that the compound had free radical scavenging activity and thus a broad spectrum was obtained. The specific compound (band) which had the anti oxidative properties showed in the R_f value of 0.77 was chosen as effective compound for antioxidant (Figure 2). The zone of inhibition observed confirmed the anti-oxidant potential of the plant. The purity of the compound was checked by TLC with 20% ethyl acetate in hexane as a single band in TLC.

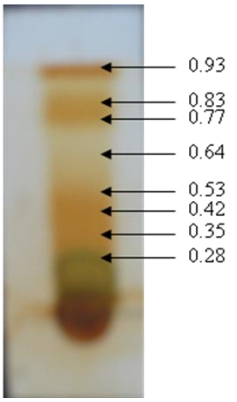

Fig. 1: Chromatogram of ethyl acetate extract of *H. pentandra* under iodine vapour

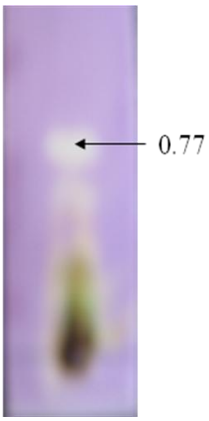

Fig. 2: Bioautography chromatogram of *H. pentandra* showing zone of inhibition at Rf 0.77

Radical scavenging activity (RSA) of *H. pentandra* plant extracts (DPPH assay)

From the dose dependent response curve of DPPH radical scavenging activity of different plant extracts of *H. pentandra,* it was observed that the ethyl acetate extract had higher radical scavenging activity than methanol and hexane. At a concentration of 160µg/ml, the scavenging activity of ethyl acetate extract reached 54%, which was comparable to that of standard. Best results of radical scavenging activity were obtained with a maximum of 66% for ethyl acetate extract which was compared to the standard α-tocopherol (84%). The values obtained were plotted in graph. The ethyl acetate plant extract of *H. pentandra* showed excellent antioxidant and free radical scavenging activity (Figure 3). In considering this, the ethyl acetate plant extract was chosen for further study.

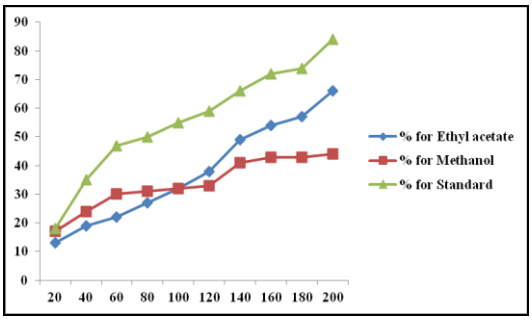

Fig. 3: DPPH Radical scavenging activity of *H. pentandra* extracts

In vitro antioxidant activity of _H. pentandra_ plant extract (FTC method)

The FTC method was used to measure the peroxide levels during the initial stage of lipid peroxidation. The OD of the sample stock and control stock was taken for 7 days. A graph was plotted between % inhibition and extract concentration. It is interesting to note that the ethyl acetate extract exhibited higher antioxidant activity than the standard α-tocopherol (Figure 4).

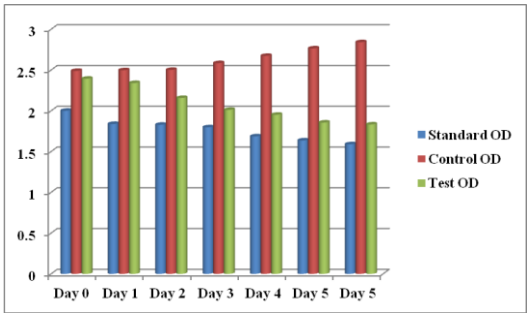

Fig. 4: _In vitro_ antioxidant activities of _H. pentandra_ plant extract (FTC method)

Hydroxyl radical scavenging activity

The hydroxyl radical is one of representative reactive oxygen species generated in the body. These radicals are produced through various biological reactions; one of the common reactions is the Iron (II)-based Fenton reaction. The scavenging capacity of the ethyl acetate extract of *H. pentandra* is shown in Figure 5. The radical scavenging capacity may be attributed to phenolic compounds in ethyl acetate extract of *H. pentandra* with the ability to accept electrons, which can combine with free radical competitively to decrease hydroxyl radical.

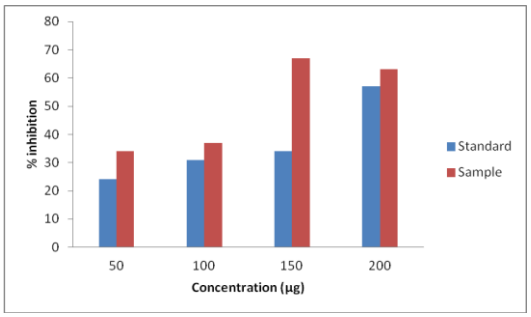

Fig. 5: Hydroxyl Radical scavenging activity of _H. pentandra_

In vitro antioxidant activity of _H. pentandra_ plant extract (TBA method)

During the oxidation process, peroxides are gradually decomposed to lower molecular weight compounds, like malonaldehyde, which can be measured by TBA method on the final day of incubation. The antioxidant activity of *H. pentandra* ethyl acetate was high on 7th day of incubation (Figure 6). The OD obtained for the sample was 0.110 and that for α-tocopherol standard was studied to be 0.098.

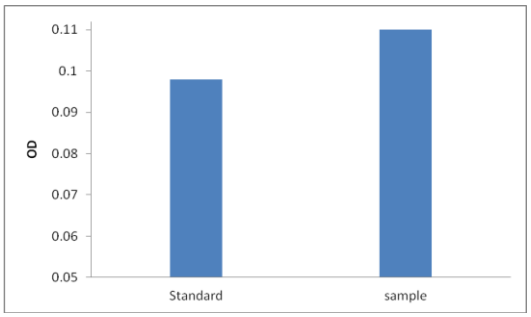

Fig. 6: _In vitro_ antioxidant activity of _H. pentandra_ by TBA method

In vitro superoxide anion radical scavenging activity of _H. pentandra_ plant extract

In this system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The reaction was initiated by the addition of PMS and the OD was taken after incubation. The decrease in absorbance at 560 nm indicates the consumption of superoxide anion in the reaction mixture. The OD of the sample (0.402) was nearly equal to that of α-tocopherol standard (0.632) as shown in figure 7. This serves as an evidence for the *in vitro* antioxidant potential of the plant.

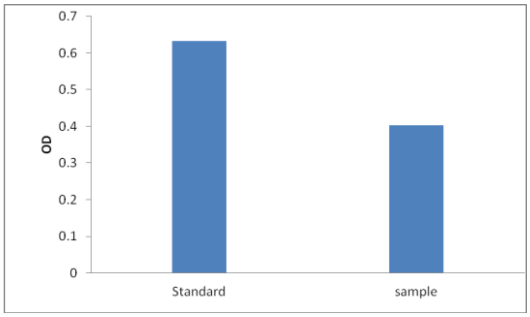

Fig. 7: _In vitro_ antioxidant activity of _H. pentandra_ by SOD method

Phosphomolybdenum assay:

The ethyl acetate extract of *H. pentandra* was analyzed in this assay to determine their antioxidant capacities by inhibiting the formation of green phosphomolybdenum complex. The results (Figure 8) indicated that the ethyl acetate extract exhibited more powerful antioxidant property by the reduction of phosphomolybdenum complex. This is evident from the data obtained where, the sample and test OD values were 87% and 73%, respectively.

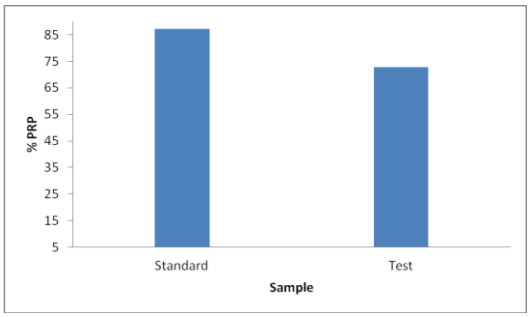

Fig. 8: Phosphomolybdenum Reducing Potential (PRP) of _H. pentandra_

Metal chelating activity

The results of metal chelating activity depicts that the ethyl acetate extract was capable of cheating metal ions. This could be proved with the % metal chelating activity values as shown in Figure 9.

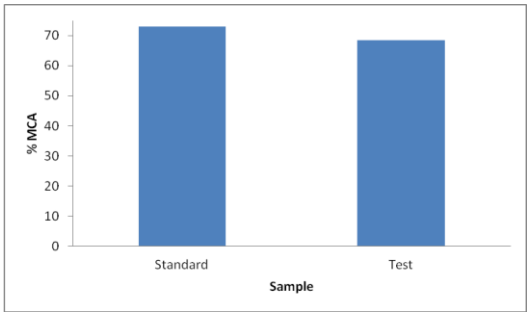

Fig. 9: Metal chelating activity of _H. pentandra_

DISCUSSION

It is a well known fact that the demand for the herbal drug treatment of various ailments is increasing and plant drugs from the Ayurvedic

system are being explored more, not only in India but also globally. As a result, many research studies are being undertaken globally [20]. A growing amount of evidence indicates a role of reactive oxygen species (ROS) such as peroxyl radicals (ROO•), hydroxyl radical (HO•), superoxide anion (O2•–) and singlet oxygen (1O2) in the pathophysiology of aging and different degenerative diseases such as cancer, cardiovascular diseases, Alzheimer's disease and Parkinson's disease. Living cells possess a protective system of antioxidants which prevents excessive formation and enables the inactivation of ROS. The antioxidants protect from the potentially damaging oxidative stress, which is a result of an imbalance between the formation of ROS and the body antioxidant defense [21,22]. In search of novel sources of antioxidants in the last years, medicinal plants have been extensively studied for their antioxidant activity [21]. Hence, the present study has investigated the phytochemical profile and the total antioxidant and free radical scavenging activities of Indian Medicinal Plant *Hydnocarpus pentandra*.

Secondary metabolites from medicinal plants function as small molecular weight antioxidants, but their particular mechanisms of action are variable. Their mechanism of action is largely based on their structure and environment, respectively. Direct anti-radical, chain-breaking of the free radical propagation, as well as interaction with transition metals can play a role. One of the mechanisms in vivo is improving the endogenous cellular antioxidant mechanisms, such as up-regulation of the activity of superoxide dismutase – (SOD) [23,24]. In the present study also, the total antioxidant and free radical scavenging activities of *H. pentandra* extracts were measured by ferric thiocyanate (FTC), thiobarbituric acid (TBA) and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) methods. It has been found that the total antioxidant and free radical scavenging activities of *H. pentandra* extracts were excellent and exhibited values closer to standard compound in activity. It is very significant that though the tested extracts were crude, their activity was comparable with standard compounds.

Phytochemical screening is an essential and very important part of medicinal plants research. Alkaloids, tannins, saponins, steroid, terpenoids, flavonoids, phenolic compounds and cardio glycoside distribution in five medicinal plants belonging to different families were assessed and compared [25]. In the present studies, the results of various phytochemical analytical methods have convincingly proved that *H. pentandra* extracts were found to have good amounts of phenolics, carbohydrates, saponins and glycosides and trace amounts of proteins.

Several studied done all over the world have proved that phenolic compounds are having exceptional antioxidant potential which makes many plants as medicinal plants [26]. The conception of antioxidant action of phenolic compounds is not novel [27]. There have been many reports of induced accumulation of phenolic compounds and peroxidase activity in plants treated with high concentrations of metals. Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper [28,29]. Various antioxidant and free radical scavenging activities carried out in the present study for the extracts of *H. pentandra* clearly indicated that the plant contains valuable medicinal properties to be used as potent antioxidant compounds. Phytochemical screening of *H. pentandra* extracts revealed very clearly that they contain good amounts of phenolics. Thus it has been concluded that the adequate presence of phenolics in *H. pentandra* makes them a potent antioxidant medicinal plant. Further analysis on the mechanism of these antioxidants and other beneficial compounds from *H. pentandra* are needed for a better understanding to implement them as potent candidates for antioxidant drugs.

CONCLUSION

The preliminary phytochemical screening of *H. pentandra* has revealed the presence of phenolics, carbohydrates, saponins and glycosides in high amounts. It is concluded that various extracts of *H. pentandra* exhibited a wide range of antioxidant capacities, thus making them a valuable source of natural antioxidants. Significant correlations were found between the antioxidant capacities and phenolic contents indicating that phenolic compounds are the major contributors of antioxidant capacity in these plant samples. Further isolation and purification of antioxidant components should be carried out for valuable utilization of these precious plant compounds. Still, further analyses on the mechanism of other beneficial compounds from various plants are needed for a better understanding to implement them into functional foods.

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