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GC-MS PROFILING OF SHOREA ROBUSTA BARK EXTRACT AND EVALUATION OF ITS ANTIOXIDANT ACTIVITY

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ABSTRACT

Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Antioxidants can also protect the human body from free radicals and ROS effects. They retard the progress of many chronic diseases as well as lipid peroxidation Also, antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. The objective of this study was to evaluate *in vitro* antioxidant, and characterize the chemical constituents of bark extract of *Shorea robusta* by using gas chromatography–mass spectrometry (GC–MS).

KEYWORDS: *Shorea robusta*, GCMS, Antioxidant, DPPH

INTRODUCTION

The Indian system of medicine has identified 1500 medicinal plants of which 500 are commonly used.[1] Phytochemistry or plant chemistry has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated with and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turn over and metabolism, their natural distribution and their biological function. Phytochemicals are the chemicals extracted from plants. These organic chemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlrophyll's etc. Secondary constituents are the remaining plant chemicals such as alkaloids (derived from amino acids), terpenes (a group of lipids) and phenolics (derived from carbohydrates).[2] Plant produces these chemicals to protect itself, but recent research demonstrates that emphasizes the plant source of most of these protective, disease-preventing compounds. A true nutritional role for phytochemicals is becoming more probable every day as research uncovers more of their remarkable benefits.[3] The aim of this paper is to determine the organic compounds present in the *Shorea robusta* bark extract with the aid of qualitative and GC-MS technique, which may provide an insight into its use in tradition medicine and to investigate the reactive oxygen and nitrogen species scavenging activity of *Shorea robusta* bark through the free radical scavenging such as superoxide anion radical scavenging, hydrogen peroxide scavenging, nitric oxide scavenging, metal chelation, reducing power activity and DPPH activity.

MATERIALS AND METHODS

GC –MS analysis

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system (Japan) comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column Elite-1 fused silica capillary column (30 x 0.25mm ID x 1µMdf, composed of 100% Dimethyl polydiloxane), operating in electron impact mode at 70eV; Helium gas (99.999%) was used as carrier gas at a constant flow of 1 ml /min and an injection volume of 0.5 µI was employed (split ratio of 10:1) injector temperature 250ºC; ion-source temperature 280ºC. The oven temperature was programmed from 110ºC (isothermal for 2 min), with an increase of 10ºC/min, to 200ºC, then 5ºC/min to 280ºC, ending with a 9min isothermal at 280ºC. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time is 36min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbomass Ver 5.2.0.

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetraacetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), DPPH (1,1-diphenyl-2-picrylhydrazyl), thiobarbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$] and L-ascorbic acid

were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials

The fully mature *Shorea robusta* barks were collected from Thanjavur District, Tamil Nadu, India from a single tree.

Preparation of alcoholic extract

The collected *Shorea robusta* barks were washed several times with distilled water to remove the traces of impurities from the leaves. The leaves were dried at room temperature and coarsely powdered. The powder was extracted with 70% ethanol for 48 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The *Shorea robusta* bark extract (SRBE) was stored in refrigerator until used. Doses such as 20, 40, 60 and 80µg/ml were chosen for in vitro antioxidant activity.

DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by the method of Shimada et al., (1992).[4] Briefly, a 2 ml aliquot of DPPH methanol solution (25 mg/ml) was added to 0.5 ml sample solution at different concentrations (20µg/ml, 40µg/ml, 60µg/ml & 80µg/ml respectively). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer. The scavenging activity of sample was expressed as 50% effective concentration (EC_{50}), which represented the concentration of sample having 50% of DPPH radical scavenging effect. The DPPH radical scavenging activity of the extract was measured according to the method of Chung et al., (2002).[5] The capability to scavenge DPPH radical was calculated by the following equation:

Scavenging effect (%) = (1–[absorbance of sample at 517 nm/absorbance of control at 517 nm]) ×100 %.

IC_{50} Value

Inhibition Concentration (IC_{50}) was introduced by Brand-Williams and his colleagues for the interpretation of the results from DPPH method.[6] The discoloration of sample was plotted against the sample concentration in order to calculate the IC_{50} value. It is defined as the amount of sample necessary to decrease the absorbance of DPPH by 50%.

Superoxide anion scavenging activity assay

The scavenging activity of the *Shorea robusta* towards superoxide anion radicals was measured by the method of Liu et al., (1997).[7] Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3ml of Tris-HCl buffer (100mmol, pH 7.4) containing 0.75ml of NBT (300µmole) solution, 0.75ml of NADH (936µmol) solution and 0.3ml of different concentrations of the SRBE. The reaction was initiated by adding 0.75 ml of PMS (120 µmol) to the mixture. After 5min of incubation at room temperature, the absorbance at 560nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

% Inhibition = [(A_0-A_1) / A_0 × 100]

Where A_0 was the absorbance of the control (blank, without SRBE) and A_1 was the absorbance in the presence of the SRBE.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction. Reaction mixture contained 60 µl of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the following equations:

% Inhibition = [(A_0-A_1) / A_0 × 100]

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method of Garrat, D.C. (1964) reported.[8] Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. finally, 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated according to the following equations:

% Inhibition = [(A_0-A_1) / A_0 × 100]

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Fe^{2+} chelating activity assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis et al., (1994).[9] To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as:

Chelating rate (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the SRBE was estimated by replacement titration.[10] Aliquot of 1.0 ml of 0.1 mmole of H_2O_2 and 1.0 ml of various concentrations of SRBE were mixed, followed by 2 drops of 3% ammonium molybdate, 10ml of 2 mole of H_2SO_4 and 7.0ml of 1.8mole KI. The mixed solution was titrated with 5.09 mmole of NaS_2O_3 until yellow colour disappeared. Percentage of scavenging of hydrogen peroxide was calculated as:

% Inhibition = $(V_0 - V_1) / V_0 \times 100$

Where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without SRBE), V_1 was the volume of NaS_2O_3 solution used in the presence of the SRBE.

Iron reducing power assay

The Fe^{3+} reducing power of the SRBE was determined by the method of Oyaizu (1986) with slight modifications.[11] The SRBE (0.75ml) at various concentrations was mixed with 0.75ml of phosphate buffer (0.2 mole, pH 6.6) and 0.75ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75ml of trichloro acetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10min. 1.5 ml of the supernatant was mixed with 1.5ml of distilled water and 0.1ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis

Tests were carried out in triplicate for 3–5 separate experiments. The amount of SRBE needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a nonlinear regression algorithm.

RESULTS AND DISCUSSION

In the present study was carried out on the plant sample revealed the presence of biologically active constituents. The phytochemical characters of the *Shorea robusta* bark were investigated and summarized in Table 1.

Table 1: Shows the Qualitative phytochemical screening of *Shorea robusta* bark.

Phytochemicals	Ethanolic extract
Tannins	Present
Flavonoids	Present
Steroids	Present
Cardiac Glycosides	Present
Terpenoids	Present
Phlobatannins	Absent
Alkaloids	Present
Anthroquinones	Present
Saponin	Absent

Identification of components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

GC-MS ANALYSIS

Seventeen compounds were identified in *Shorea robusta* bark by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 2 and Fig.2. GC-MS Studies of SRBE indicates that the prevailing compounds were Oleanane-type terpenoid fractions as α-amyrin (RT-34.69), Triterpenoids fraction of β-amyrin (RT-33.55), Sesquiterpenoids fraction as Caryophllene oxide (RT-8.83) and steroids fraction as Stigmastan-2,22 dien, 3,5, dedihyro (RT 31.41). The qualitative analysis of *Shorea robusta* bark extract contains flavonoids, alkaloids, tannin, steroids, terpenoids present in it.

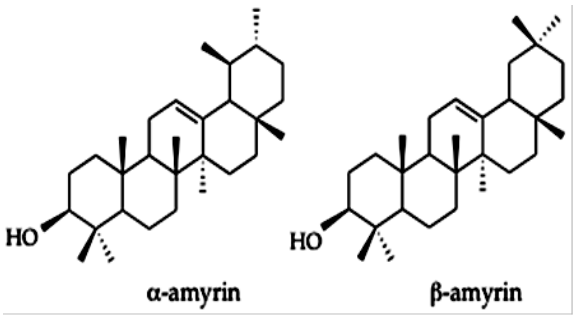

Fig. 1: Shows the structure of α and β amyrin.

α and β amyrins have been shown to exhibit various pharmacological actitivies *in vitro* and *in vivo* conditions against various health-related conditions, including conditions such as inflammation, antimicrobial, fungal and viral infections, anti-ulcer, cancer cells, antinociceptive, antioxidant, antipruritic and hepatoprotective effects.

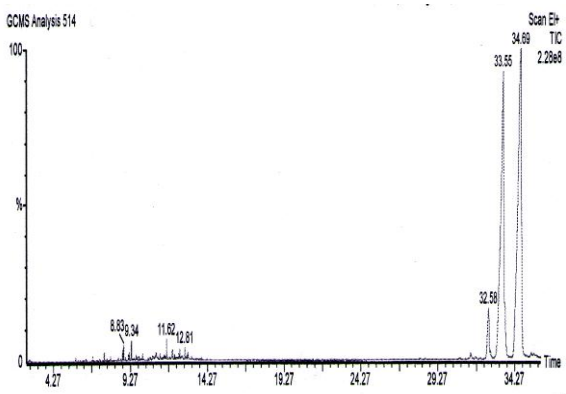

Fig. 2: Chromatogram obtained from the GC/MS with the extract of *Shorea robusta* bark.

Table 2: Shows the components identified in ethanolic extract of *Shorea robusta* bark (GC MS study).

*Components identified in the Shorea rebusta sample - 105

[GC MS study]

No.	RT	Name of the compound	Molecular formula	MW	Peak Area %
1	2.71	Propane, 1,1,3-triethoxy-	$C_9H_{20}O_3$	176	0.04
2	5.71	Cyclohexene, 4-ethenyl-4-methyl-3-[1-methylethenyl]-1-[1-methylethyl]-, [3R-trans]-	$C_{15}H_{24}$	204	0.05
3	6.40	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis[1-methylethenyl]-	$C_{15}H_{24}$	204	0.04
4	6.83	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (Z)- [Synonyms: (Z)-ò-Farnesene]	$C_{15}H_{24}$	204	0.06
5	7.57	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-[1-methylethyl]-, [s-[E,E]]- [Synonyms: Germacrene D]	$C_{15}H_{24}$	204	0.11
6	7.98	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-[1-methylethyl]-, (1S-cis)- [Synonyms: Cadina-1(10),4-diene]	$C_{15}H_{24}$	204	0.66
7	8.83	Caryophyllene oxide	$C_{15}H_{24}O$	220	0.20
8	9.16	endo-2-Methylbicyclo[3.3.1]nonane	$C_{10}H_{18}$	138	0.09
9	9.34	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aà,4aà,7à,7aà,7bà)]- [Synonyms: Spathulenol]	$C_{15}H_{24}O$	220	0.31
10	11.62	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl-	$C_{15}H_{26}$	206	0.28
11	12.01	trans-2-ò-Bisabolene epoxide	$C_{15}H_{24}O$	220	0.22
12	12.81	1-Cyclohexene-1-butanal, à,2,6,6-tetramethyl-	$C_{14}H_{24}O$	208	0.38
13	13.02	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	0.14
14	31.41	Stigmastan-6,22-dien, 3,5-dedihydro-	$C_{29}H_{46}$	394	0.34
15	32.58	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	$C_{30}H_{48}O$	424	4.36
16	33.55	B-Amyrin	$C_{30}H_{50}O$	426	42.90
17	34.69	a-Amyrin	$C_{30}H_{50}O$	426	49.79

In recent years, focus on plant research has increased all over the world and a large number of evidences have been collected to show the immense potential of medicinal plants used in traditional systems. Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities, including antioxidant potential. Natural antioxidants are in high demand for application as nutraceuticals, bio-pharmaceuticals, as well as the food additive because of consumer preference. A number of methods and variations have been developed and applied for the measurement of antioxidant capacity and efficacy.[12] The plants and its derivatives may considered as good sources of natural antioxidants for medicinal uses such as against cancer, diabetic mellitus, cardiovascular diseases, aging and other diseases related to radical mechanisms. Plant derived antioxidant therapy may be helpful for various free radical mediated diseases. Recently importance has been given for *in vitro* antioxidant study to understand the pharmacological role of medicinal plant. *In vitro* techniques have been used for detection of antioxidants, which are based on the ability of compounds to scavenge peroxy radicals.[13]

DPPH Radical scavenging activity

Free radicals are harmful by-products generated during normal cellular metabolism, which could initiate oxidative damage to body.[14] Antioxidants are believed to play a significant role in the body's defense system against free radicals. Recently, numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, vegetables, herbs and cereals extracts. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants.[15]

The DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The half inhibition concentration (IC_{50}) of *Shorea robusta* was 0.58 µg ml^{-1}**.** The antioxidant activity of SRBE *is* shown in Fig.3. The SRBE exhibited a significant dose dependent inhibition of DPPH activity.

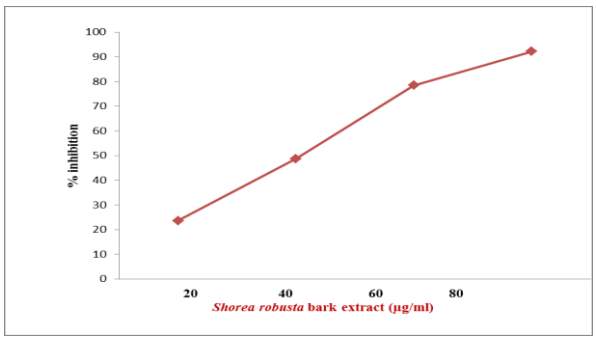

Fig. 3 Shows DPPH oxide radical scavenging activity of *Shorea robusta* at different concentrations.

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system.[16] The superoxide anion radical scavenging activity *Shorea robusta* assayed by the PMS-NADH system is shown in Fig.4. The superoxide scavenging activity of *Shorea robusta* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of *Shorea robusta* was 0.42 µg ml^{-1}. These results suggested that *Shorea robusta* had superior superoxide radical scavenging effect.

Hydroxyl radical scavenging activity

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. Fig.4 showed the *Shorea robusta* exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC_{50} of *Shorea robusta* was 0.49 µg ml^{-1}. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxy substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups.

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effect or molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities.[19] *Shorea robusta* extract also moderately inhibited nitric oxide in dose dependent manner (Fig. 4) with the IC_{50} being 0.42 µg ml^{-1}.

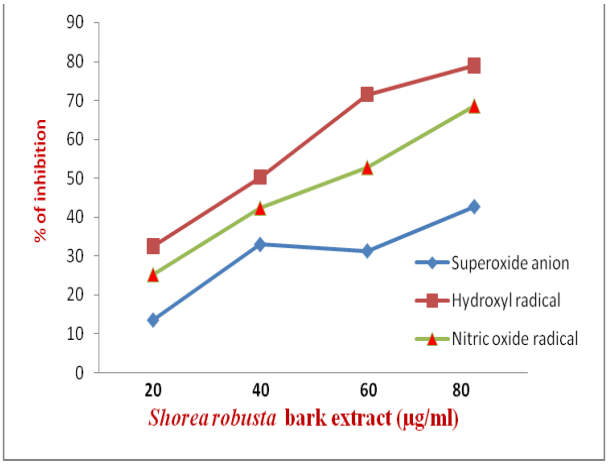

Fig. 4 Shows Superoxide, Hydroxyl and Nitric oxide radical scavenging activity of *Shorea robusta* at different concentrations

The ferrous ion chelating activity

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red coloured) formation is interrupted and as a result, the red colour of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of colour reduction. The formation of the ferrozine Fe^{2+} complex is interrupted in the presence of aqueous extract of *Shorea robusta*, indicating that have chelating activity with an IC_{50} of 0.47 µg ml^{-1} (Fig.5). Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals.[17] Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that form s bonds with a metal are effective as secondary antioxidants because they reduce the redox potential and thereby stabilize the oxidized form of the metal ion.[18] Thus, *Shorea robusta* demonstrate a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects.[14] It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. As shown in Fig. 5, *Shorea robusta* demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC_{50} of 0.39 µg ml^{-1}.

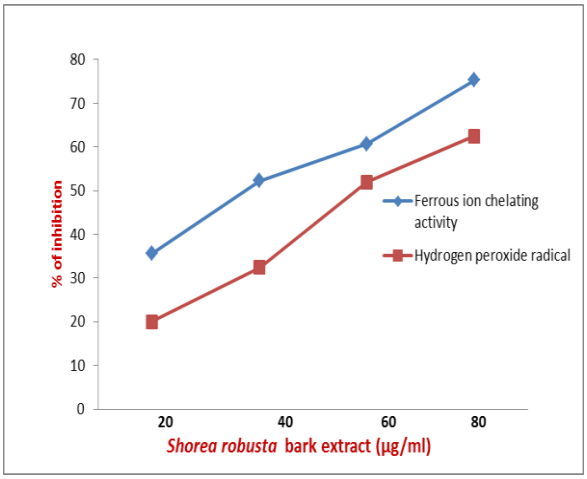

Fig. 5 Shows hydrogen peroxide and ferrous ion chelating activity of *Shorea robusta* at different concentrations.

Iron reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *Shorea robusta*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging.[20,21] Fig. 6 depicts the reductive effect of *Shorea robusta*. Similar to the antioxidant activity, the reducing power of *Shorea robusta* increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that *Shorea robusta* consist of

hydrophilic polyphenolic compounds that cause the greater reducing power.

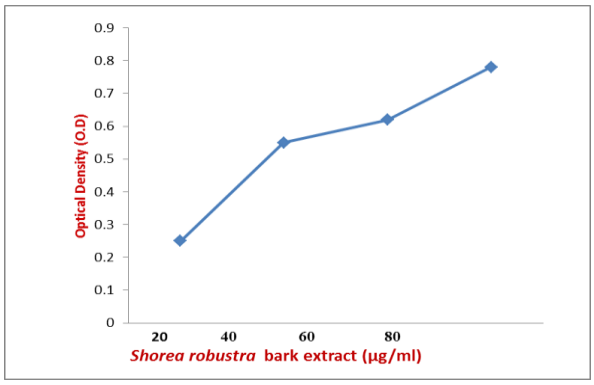

Fig. 6: Shows the Reducing power activity of *Shorea robusta* bark at different concentrations.

CONCLUSION

The SRBE showed that strong antioxidant activity through DPPH, iron reducing power activity, superoxide anion scavenging and hydrogen peroxide scavenging activities. This work has gathered experimental evidence on the SRBE as natural antioxidant for its capacity to scavenge reactive oxygen species/free radicals and protect cells/organism from oxidative damage and thus could be an effective against oxidative stress. In addition, the SRBE found to contain a noticeable amount of α and β amyrins which plays a major role in controlling antioxidants. Thus, it can be concluded that SRBE can be used as an accessible source of natural antioxidants with consequent health benefits.

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REFERENCES

1. Agrawal OP, Raju PS. Global market of herbal products: Opportunities for Indian Traditional System of Medicine. New Delhi, India, Narcosa Publishing House, 2006; 5-10.
2. Liu RH. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. Journal of Nutrition, 2004; 134(12 Suppl.): 3479S–3485S.
3. Hamburger M, Hostettmann, K. Bioactivity in plants: the link between phytochemistry and medicine. Phytochemistry, 1991; 30: 3864-74.
4. Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T Antioxidative properties of xanthum on the autoxidation of soybean oil in cyclodextrin emulsion. Journal of Agricultural and Food Chemistry, 1992; 40: 945–948.
5. Chung KT, Wong T., Wei CI and Huang YW Tannins and human health: a review Critical Review in Food Science and Nutrition, 1998; 38: 421.
6. Brand-Williams W, Cuvelier ME and Berset C. Use of free radical method to evaluate antioxidant activity. Lebensmittel Wissenschaft and Technologie 1995; 28: 25-30.
7. Liu F, Ooi VEC, Chang ST Free radical scavenging activity of mushroom polysaccharide extracts. Life Sci., 1997; 60: 763-771.
8. Garrat DC The quantitative analysis of drugs. Chapman and Hall ltd, Japan. Biochem. Anal. Chem., 1964; 3: 456-458.
9. Dinis TCP, Madeira VMC, Almeidam LM Action of phenolic derivates (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and peroxyl radicals scavegers. Achieves of Biochemistry and Biophysics, 1994; 315: 161-169.
10. Zhang X.Y. Science Press; Beijing, China: Principle of Chemical Analysis, 2000; 275–276.
11. Oyaizu M. Studies on products of browning reactions: antioxidant activities of products of browning reaction prepared from glucose amine. Japanese Journal of Nutrition, 1986; 44: 307-315.
12. Harborne JB Phytochemical methods, London. Chapman and Hall, Ltd., 1973; 49-188.
13. Velavan S, Nagulendran K, Mahesh R In vitro antioxidant activity of *Asparagus racemosuss* root. Pharmacog. Magaz., 2007; 3: 26-33
14. Halliwell B, Gutteridge JMC The definition and measurement of antioxidants in biological systems. Free Radic Biol Med., 1995; 18: 125–126.
15. Nuutila AM, Puupponen-Pimiä R, Aarni M. and Oksman-Caldentey K M. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. Food Chemistry, 2003; 81: 485–493.
16. Korycka-Dahl M, Richardson M Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and aminoacids. Journal of Dairy Science, 1978; 61: 400-407.
17. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. The American Journal of Medicine, 1991; 91: S14-S22.
18. Gordon MH The mechanism of the antioxidant action in vitro. In B. J. F. Hudson, Food Antioxidants, London: Elsevier., 1990; 1-18.
19. Miller MJ, Sadowska-krowicka H, Chotinaruemol S, Kakkis JL, Clark DA Amelioration of chronic ileitis by nitric oxide synthase inhibition. J. Pharmac. and Experi. Thera. 1993; 264: 11-16.
20. Diplock AT Will the 'good fairies' please prove to us that vitamin E lessens human degenerative disease? Free Radical Research, 1997; 27: 511-532.
21. Yildirim A, Mavi A, Oktay M, Kara AA, Algur OF, Bilaloglu V Comparison of antioxidant and antimicrobial activities of Tilia (Tilia argentea Desf Ex DC), Sage (Salvia triloba L.), and Black Tea (Camellia sinensis) extracts. Journal of Agricultural and Food Chemistry, 2000; 48: 5030-5034.