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Evaluation of *In Vitro* & *In Vivo* Antioxidant Studies of Whole Plant of 70% Ethanolic Extracts of *Indigofera Barberi Gambles*

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ABSTRACT

The aim of the present work is to evaluate the *in vitro* and *in vivo* antioxidant activity of 70% ethanolic extract of the whole plant of Indigofera barberi. *In vitro* antioxidant activity was evaluated by studying DPPH radical scavenging activity, nitric oxide scavenging activity, hydrogen peroxide scavenging activity and metal chelating activity. Ascorbic acid and butylated hydroxyl toluene were used as standards for all the experiments; invivo antioxidant activity was evaluated by LPO, SOD, CAT & GSH. IC_{50} values observed for studying DPPH radical scavenging activity, nitric oxide scavenging activity, hydrogen peroxide scavenging activity and metal chelating activity were determined to be 274.44, 283.24, 328.68 & 341.58 .Each experiment was carried out in triplicate and results are expressed as mean ± S.E.M. It showed the increased activity of invivo antioxidant enzymes when compared to negative control animals. The results clearly indicated that ethanolic extract of whole plant of *Indigofera barberi* of the study species is effective in scavenging free radicals and has the potential to be a powerful as invitro and invivo antioxidant.

Keywords: *In vitro* antioxidant activity, *Boswellia ovalifoliolata Linn*, DPPH, SOD, CAT.

INTRODUCTION

Oxidative stress occurs when there is excessive free radical production and/or low antioxidant defense, which leads to chemical alterations of bio molecules causing structural and functional modifications. Free radicals, which have one or more unpaired electrons, are produced in normal or pathological cell metabolism. Reactive oxygen species (ROS) react easily with free radicals to become radicals themselves. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals ($O2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$), as well as non-free radical species (H_2O_2) and the singled oxygen ($^1O_2$) (Ilhami Gulcina et al., 2003) **.** Reactive oxygen species (ROS) are considered to be important factors in etiology of several pathological conditions (Ravi kumar R & Kalidoss A, 2014). When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases (Gutteridge JMC, 1995). Two principle mechanisms of action have been proposed for antioxidants. The first is a chain breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky NI, 1992). There are two types of antioxidant are available i.e. In vitro antioxidants & In vivo antioxidants.

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Indigofera Barberi Gambles

Indigofera is a large genus of over 750 species of flowering plants belonging to the family Fabaceae. It consists of dried whole plant known as *Indigofera barberi* Gamble. The telugu names are Thambajalari & Thummajalari.

They are widely distributed throughout the tropical and subtropical regions of the world (Wills JCA, 1985). The species are mostly shrubs, through some are herbaceous and few can become small trees up to 5-6 m tall. Most are dry season or winter deciduous. *Indigofera barberi* Gamble is a rare, endemic plant mainly found in Shesha chalam hills. The population of this plant species

is rapidly declining due to various factors such as habitat destruction, fragmentation of population and lower percentage of seed germination etc. Traditional uses are the entire plants including flowers of this plant are well known in treating jaundice and nephroprotective action (Palani S, 2008). The entire plant used in the treatment of wounds, cuts, boils, burns, skin diseases, jaundice, anthelmintic and diabetes (Madhav chetty K et al., 2008). These plants are also used in the treatment of certain diseases like urolithiasis, arthritis & hypertension.

MATERIALS AND METHODS

Chemicals and Standards

DPPH (1,1-diphenyl-2-picryl hydrazyl), ascorbic acid, BHT, (Butylated hydroxyl toluene), adrenaline, GSH were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (petroleum ether , chloroform, ethyl acetate, ethanol,), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride, , hydrochloric acid, copper sulphate, aluminum chloride, lead acetate, acetic acid & ammonia from Merck (Pvt.) Ltd. (Germany).

Plant material

The fresh bark of Boswellia ovalifoliolata were collected in December 2009 from sesachala hills of Tirupati, Andhra Pradesh (India) and authenticated by Dr. K. Madhav Chetty, Assistant Professor, Taxonomist, Dept. of Botany, Sri Venkateswara University, Tirupathi, Andhra Pradesh 20.A voucher specimen has been kept in our laboratory for future reference

Preparation of extract

The whole plant was cleaned, air dried and grounded into powdered. The dried powdered plant material was passed through sieve 40 and stored in air tight containers. The powdered material was subjected to successive solvent extraction using Benzene, chloroform, ethyl acetate, ethanol & water. Initially 100gm of crude powder was taken and packed in a packing paper. This pack was placed in a soxhlet extractor for extraction with different solvents i.e. (Chloroform, ethyl acetate, ethanol& water) and the temperature was adjusted as per the solvent been used in the extraction. The extract is then concentrated and dried under reduced pressure. The percentage yield obtained was calculated and reported (Khandelwal KR, 2005).

The percentage yield of the extract was calculated by using the following formula:

$$\% \text{ yield} = \frac{\text{Weight of the extract}}{\text{Weight of plant material}} \times \frac{100}{1}$$

Preliminary phytochemical analysis

The preliminary phytochemical studies were conducted on the active extracts using standard procedures adopted by Harborne (1973) and Gibbs (1974). Preliminary phytochemical analysis on plant extracts was performed using the following chemicals and reagents: flavonoids (Mg metal and HCl), phenolics ($FeCl_3$), protein and amino acid (Millon's and Ninhydrin reagent), alkaloids (Mayer and Dragendorff's reagent), saponins (Foam test), phytosterols, triterpenoids (Liebermann- Burchard Test) and carbohydrates (Fehling's solution A and B) (Harborne JB, 1973; Kokate CK et al., 2009).

Experimental animals

Animal Husbandry

The experiment protocol described in the present study was approved by the institutional animal ethical committee (IAEC) and with the permission from committee for the purpose of control and supervision of experiments on animals (CPCSEA) ministry of social justice and empowerment, government of India. Reg No. IAEC/1521/PO/a/11/CPCSEA. Healthy adult male wistar rats weighing 150- 200g were used. Rats were acclimatized to laboratory conditions one week prior to initiation of experiments. Rats were housed in poly propylene cages, maintained under standardized condition which means that 12: 12 hour light/dark cycle in polypropylene cages, 24±2°C, and 50 ± 5 % humidity. Standard pellet feed (Hindustan Lever Limited., Bangalore) and drinking water was provided ad libidum throughout experimentation period. The animals were not provided food for 12 hour before experimentation but allowed free access to water throughout**.**

Acute toxic class method

Alcoholic extracts were studied for the acute oral toxicity according to the guidelines set by Organization for Economic Co-operation and Development (OECD) guidelines number 423. The acute oral toxicity study was carried out as per the guild lines said by OECD received draft guidelines 423 (acute toxic class method, Ecobichon, 1997) (OECD guidelines 2008) received from CPCSEA, ministry of social justice and empowerment, government of India.

Determination of antioxidant activity

The antioxidant activity was evaluated by five ways which are as follows;

Free radical scavenging activity (DPPH method)

The scavenging activity for DPPH free radicals was measured according to the procedure described by Blios *et al*, 1958. Ethanolic solution of the sample extract at various concentrations (50, 100, 150, 200 and 250 ug/mL) was added separately to each 5 mL of 0.1 m M methanolic solution of DPPH and allowed to stand for 20 min at 27c. After incubation, the absorbance of each solution was determined at 517 nm using spectrophotometer. Ascorbic acid & BHT (butylated hydroxy toluene) was used as standard. The corresponding blank reading was also taken and DPPH radical scavenging activity was calculated by using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} * 100$$

IC_{50} value is the concentration of the sample required to scavenge the 50% DPPH free radical.

Nitric oxide scavenging capacity assay

Nitric oxide scavenging assay was carried out using sodium nitroprusside(Sreejayan N and Rao MNA, 1997). This can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract/sub-fraction at various concentrations and the mixture was incubated at 25°C for 150 min. From the incubated mixture, 0.5 ml was taken out and added into 1.0 ml sulphanilamide solution (0.33% in 20% glacial acetic acid) and further incubated at room temperature for 5 min. Finally, 1.0 ml naphthyl ethylene diamine hydrochloride (0.1% w/v) was mixed and maintained at room temperature for 30 min. The absorbance was measured at 546 nm. A typical blank/control solution contained the same solution mixture without plant extract or standard. The absorbance of the blank/control solution was measured at 546 nm.

The percentage inhibition was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} * 100$$

Scavenging of hydrogen peroxide

Scavenging activity of extract and its sub-fractions were evaluated by hydrogen peroxide (Jaya prakasha GK et a., 2004), 1 ml of various concentrations of the extract, sub-fractions and standards in ethanol was added to 2 ml of hydrogen peroxide solution in phosphate buffered saline (PBS, pH 7.4). Then finally the absorbance was measured at 230 nm after 10 min. Ascorbic acid and BHT were used as standard. Control sample was prepared containing the same volume without any extract and standard and the absorbance were read at 230 nm using a spectrophotometer. The percentage inhibition was calculated according to the following equation: Hydrogen peroxide scavenging activity (%) was calculated as:

The percentage inhibition was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} * 100$$

Metal chelating activity

The chelating of ferrous ions by the extract of stem of Boswellia ovalifoliolata was estimated by the method described by Dinis TCP et al, 1994). Various concentrations of the extracts viz., 50, 100, 150, 200, 250 and 300 ug/mL of sample was added with 1 ml of 2mM FeCl2 separately. The reaction was initiated by the addition of 5mMferrozine (1ml). Absorbance was measured at 562nm after 10min. Ascorbic acid and BHT was used as standards.

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} * 100$$

Measurement of antioxidant enzymes

Liver tissues were homogenized in four volumes of ice-cold 150 mM Tris–HCl (pH 7.4) using homogenizer. The homogenates were centrifuged at 1600g for 15 min at 4^0 C to obtain a supernatant for various biochemical analyses.

Measurement of Lipid peroxidation (LPO)

The concentration of TBA reactive substances was measured (lipid peroxidation product malondialdehyde MDA was estimated) in liver using the method of Okhawa H et al., 1979).

METHOD

1 ml of the sample was mixed with 0.2 ml 4% (w/v) Sodium dodecyl sulphate, 1.5 ml 20% acetic acid in 0.27 M hydrochloric acid (pH- 3.5) and 15 ml of 0.8% TBA (pH- 7.5). The mixture was heated in a hot water bath at 25^0C for one hour. The intensity of the pink color developed was read against a reagent blank at λ_{max} 532 nm.The percentage of inhibition of lipid peroxidation was calculated by comparing the results of test with those of control not treated with extracts.

Super oxide dismutase (SOD)

SOD was estimated by the method of Misra and Fridovich (1972)

Principle

Rate of auto oxidation of epinephrine and the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8 -10.2, O_2 generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced. The auto oxidation of epinephrine proceeds by at least two distinct pathways only one of which is free radical chain reaction involving O_2 and hence inhabitable by SOD.

Reagents

1. Carbonate buffer (0.05 M, pH 10.2): 16.8 g. of sodium bicarbonate and 22 g of sodium carbonate were dissolved in 500 ml of distilled water and final volume was made up to with distilled water.
2. Ethylene diamine tetra acetic acid (EDTA) (0.49 M): 1.82 g of EDTA was dissolved in 1000 ml of distilled water.
3. Epinephrine (3 mM): 9.9 mg of epinephrine bitartrate was dissolved in 10 ml of 1M HCl solution.
4. SOD standard: dissolve 1mg (1000 units/mg) of SOD from bovine liver in 100 ml of carbonate buffer.

Procedure

0.5ml of sample was diluted with 0.5 ml distilled water, to this 0.25 ml ethanol, 0.5 ml of chloroform (all chilled reagents) were added. The mixture was shaken for 1 min and centrifuged at 200 rpm

for 20 min. The enzymatic activity of supernatant was determined to it 0.05 ml of carbonate buffer (0.05 M pH 10.2) and 0.5 ml EDTA (0.49 M) was added. The reaction was initiated by addition of 0.4 ml epinephrine and the change in optical density/mm was measured at 480 nm. SOD was expressed as units/mg protein change in optical density/mm 50% inhibition of epinephrine to adrenochrome transition by enzyme is taken the enzyme unit. Calibration curve was prepared using 10-125 units SOD.

$$SOD = \frac{(0.025\text{-}Y)}{Y \times 50} \times 100$$

Y- Final reading – initial reading

Catalase (CAT)

Catalase was estimated by the method of Hugo E. Aebi E, 1984.

Principle

In UV range H_2O_2 can be followed directly by the decrease in absorbence (O.D 240) per unit time is measure of catalase activity.

$H_2O_2 \longrightarrow H_2 + O_2$

$RDOH \longrightarrow H_2O + ROH + A$

Decomposition of H_2O_2 = Decrease in absorbance at 240 nm

Reagents

1. Phosphate buffer (50 mM, pH7.0)

A. Dissolve 6.81 g KH_2PO_4 in distilled water and make up to 1000 ml.

B. Dissolve 8.9 g NaH_2PO_4. $2H_2O$ in distilled water and make up to 1000 ml.

Mix the solution Aand B in proportion 1:15 (v/v)

2. Hydrogen peroxide (30 mM/I): Dilute 0.34 ml of 30% Hydrogen peroxide with phosphate buffer up to 100 ml.

Procedure

Dilute homogenate 20 times with Phosphate buffer pH 7.0

Blank	Test
4 ml of homogenate diluted with 2 ml of phosphate buffer P^H 7, and take absorbance at 254 nm for 3 min. with 30 sec interval.	2 ml of homogenate diluted with 1 ml of H_2O_2 (8.5 micro lit. in 2.5 ml phosphate buffer (50mM/l. pH 7.0) and take the absorbance at 254 nm for 3 min. with 30 sec. interval. (Add H_2O_2 just before taking O.D)

Calculation

Log (A / B) × 2297.3

Where,

A: Initial absorbance

B: final absorbance (after 30 second)

Units = µ moles of H_2O_2 consumed/min/mg

Estimation of Reduced glutathione (GSH)

Reduced glutathione was determined by the method of Moran MS *et al*., 1979.

Reagents

1. TCA (10% w/v) solution: Accurately weighed 10 g of TCA was dissolved in 100 ml of distilled water.
2. Phosphate buffer (0.2 M, pH 8)
3. DTNB reagent (0.6 M): 60 mg of 5, 5- dithio bis (2-nitro benzoic acid) was dissolved in 100 ml of 0.2 M sodium phosphate (pH8).
4. Standard glutathione: Prepared by dissolving 10 mg of reduced glutathione in 100 ml of distilled water.

Procedure

To 1 ml of sample, 1 ml of 10% TCA was added. The precipitated fraction was centrifuged and to 0.5 ml supernatant, 2 ml DTNB was added. The final volume was made up to 3 ml with phosphate buffer. The colour developed was read at 412 nm. The amount of glutathione was expressed as µg of GSH/mg protein reduced glutathione was used as standard (100µg/ml).

$$X = \frac{(Y - 0.0046)}{0.0034}$$

Y – Absorbance of test sample

Statistical analysis

Data were expressed as Mean ± SEM. Linear regression analysis was used to calculate the IC50 value. Student's t-test was used for the comparison between two means for the possible significant interrelation. Data were considered statistically significant only when P value < 0.05

RESULTS

Percentage yield of ethanolic extracts of Indigofera barberi (whole Plant)

% yields of 70% ethanolic extracts of Indigofera barberi

Part used: Whole plant

Weight of dried leaves: 1kgs

Extracted with: Chloroform, ethyl acetate, 70% ethanol &water.

% yield of 70% IBEE: 7.60%

Preliminary Phytochemical Screening

The results obtained from the preliminary phytochemical investigations of different extracts of whole plant of *Indigofera barberi* were summarized in Table.2.

Glycosides, carbohydrates, flavonoids, tannins & phenols were present in chloroform extract. Glycosides, carbohydrates, Flavonoids, steroids, tri-terpenoids, tannins & phenols were present in ethyl acetate extract. Glycosides, Carbohydrates, Flavonoids, steroids, tri-terpenoids, tannins & phenols were present in 70% ethanol extract. Flavonoids, tannins & phenols were present in aqueous extract. Alkaloids, Proteins, amino acids&Saponins were absent in all the extracts whereas glycosides, flavonoids, tannins and phenols were present in all the extracts.

Table.1 Percentage of yield of different extracts of Boswellia ovalifoliolata (bark)A, Indigofera barberi (whole plant) B& Rhynchosia beddomei(leaves)C, With different solvents

S No	Parameters	Chloroform	Ethyl acetate	70% Ethanol	Water
1	Consistency	Oily	Oily	Viscous	Viscous
2	Colour	Green	Brownish green	Radish black	Cream
3	% of yield	4.75%	3.50%	7.60%	4.20%

Table.2 Preliminary phytochemical screening of the different extract of *Indigofera barberi*

S No	Phyto constituents	Chloroform	Ethyl acetate	Ethanol	Water
1	Alkaloids	-	-	-	-
2	Glycosides	+	+	+	-
3	Carbohydrates	+	+	+	-
4	Proteins	-	-	-	-
5	Amino acids	-	-	-	-
6	Flavonoids	+	+	+	+
7	Steroids	-	+	+	-
8	Tri-terpenoids	-	+	+	-
9	Tannins	+	+	+	+
10	Phenols	+	+	+	+
11	Saponins	-	-	-	-

Where, + = Presence, − = Absence

Table.3 Acute toxicity studies of the 70% ethanolic extract of *Indigofera barberi (IBEE)*

Group	Dose	No. of rats	Mortality	
			24 hrs	14 days
Group -I	50 mg/kg b.wt(70% *IBEE*)	3	0	0
Group -II	300 mg/kg b.wt(70% *IBEE*)	3	0	0
Group -III	2000 mg/kg b.wt(70% *IBEE*)	3	0	0
Group -IV	4000 mg/kg b.wt(70% *IBEE*)	3	0	0

Table.4 The absorbance and scavenging activity or % inhibition of DPPH by70% *IBEE*

Conc. µg/ml	*Indigofera barberi*	Ascorbic acid	BHT
50	15.38±0.65	20.12±0.51	18.16±0.99
100	19.25±0.96	28.12±0.89	21.36±1.11
150	28.36±1.21	39.46±1.16	33.16±1.32
200	37.34±1.75	56.23±1.52	47.19±1.58
250	45.28±1.84	79.16±1.98	72.38±1.81
IC50	274.44	168.62	193.32

Table.5 The absorbance and scavenging activity or % inhibition of NO by 70% *IBEE*)

Conc. µg/ml	*Indigofera barberi*	Ascorbic acid	BHT
50	16.18±0.46	28.36±0.76	20.17±0.75
100	22.39±0.94	39.38±0.89	31.25±0.91
150	32.25±1.98	48.16±1.13	42.36±1.27
200	39.38±1.56	59.24±1.09	53.19±1.65
250	44.16±1.71	70.36±1.89	64.29±1.17
300	49.06±1.32	84.29±1.87	72.39±1.69
IC50	283.24	161.33	190.55

Table.6 The absorbance and scavenging activity or % inhibition of H202 by 70% *IBEE*

Conc. µg/ml	*Indigofera barberi*	Ascorbic acid	BHT
50	10.11±0.38	26.48±0.63	18.36±0.95
100	19.25±0.70	39.38±0.74	27.42±0.56
150	24.36±1.36	47.19±1.64	34.28±1.74
200	32.28±0.71	59.28±2.11	42.36±1.36
250	38.39±0.89	70.38±2.81	62.38±1.64
300	44.65±2.11	84.09±1.36	76.43±1.48
IC50	328.68	162.9	203.46

Table.7 % inhibition of metal chelating activity by 70% *IBEE*

Conc. µg/ml	*Indigofera barberi*	Ascorbic acid	BHT
50	12.74±0.68	28.32±1.24	**24.06**±0.59
100	19.36±0.91	39.38±0.96	31.25±0.83
150	27.36±1.69	48.12±1.58	40.39±1.72
200	32.42±1.39	52.64±1.63	51.13±1.69
250	36.35±2.72	69.26±2.81	62.19±2.74
300	42.36±2.81	84.36±2.11	77.23±1.72
IC50	341.58	166.05	188.86

Table.8 Effect of graded doses of IBEE on anti-oxidant enzymes in CCl_4 induced hepatotoxicity model

Groups	Dose (mg/kg)	LPO (µg/mg prot)	CAT (µg/mg prot)	SOD (µ/mg prot)	GSH (µg GSH/ prot)
I (Normal Control)	-	42.58 ± 3.15	82.37±3.72	14.93 ± 0.86	1.21 ± 0.09
II (CCl_4 control)	-	75.89 ± 4.75	30.66±1.85	5.16 ± 0.61	0.39± 0.06
III (Standard)	Silymarin 50	49.42 ± 4.32^c	75.76± 6.62^c	12.49 ±0.91^c	1.04± 0.09^c
IV (Test -1)	BOEE 100	60.71 ± 4.94	52.58±2.98^a	6.33 ±0.86	0.59± 0.08
V (Test -2)	BOEE 200	56.25 ± 3.54 ^{b}	64.74±3.07^c	8.25 ±0.92^a	0.81± 0.08^b
VI (Test -3)	BOEE 400	52.85 ± 3.48^b	71.62±3.48^c	10.90 ±0.61^c	0.90±0.07^c

Data represent the Mean ± SEM, (n=6); Statistical significance: ^{a}P< 0.05, ^{b}P< 0.01, ^{c} P < 0.001, versus with respect to CCl_4 control groups (One way ANNOVA followed by Dunnet's multiple comparison test)

Figure.1 DPPH scavenging activity of *70% BOEE (A), IBEE (B) & RBEE(C)*

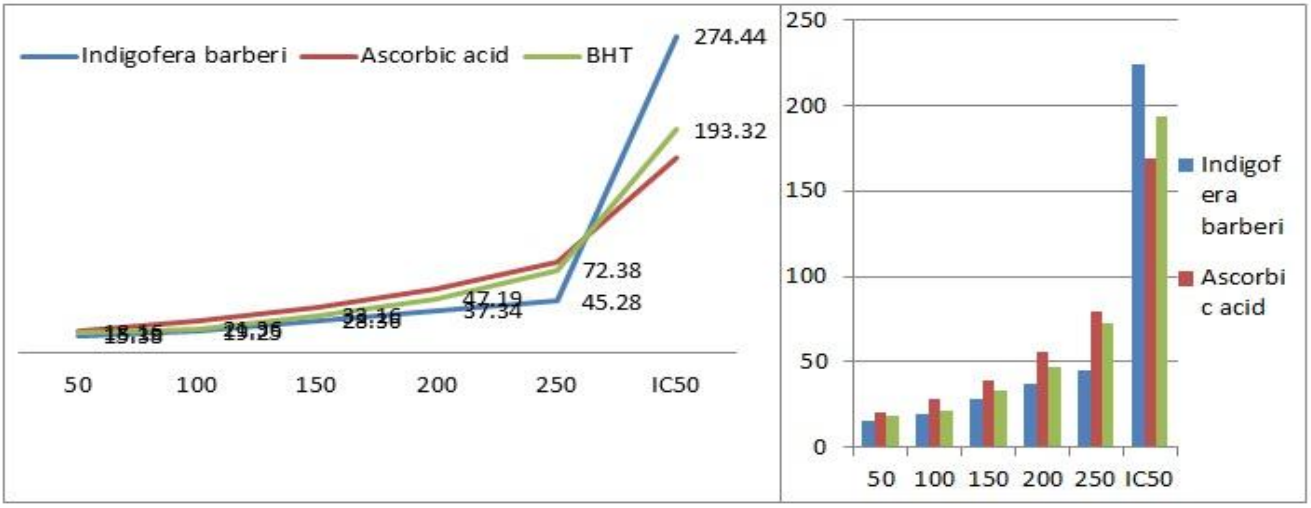


Figure.2 Graphical representation of % inhibition of NO by 70% *IBE*

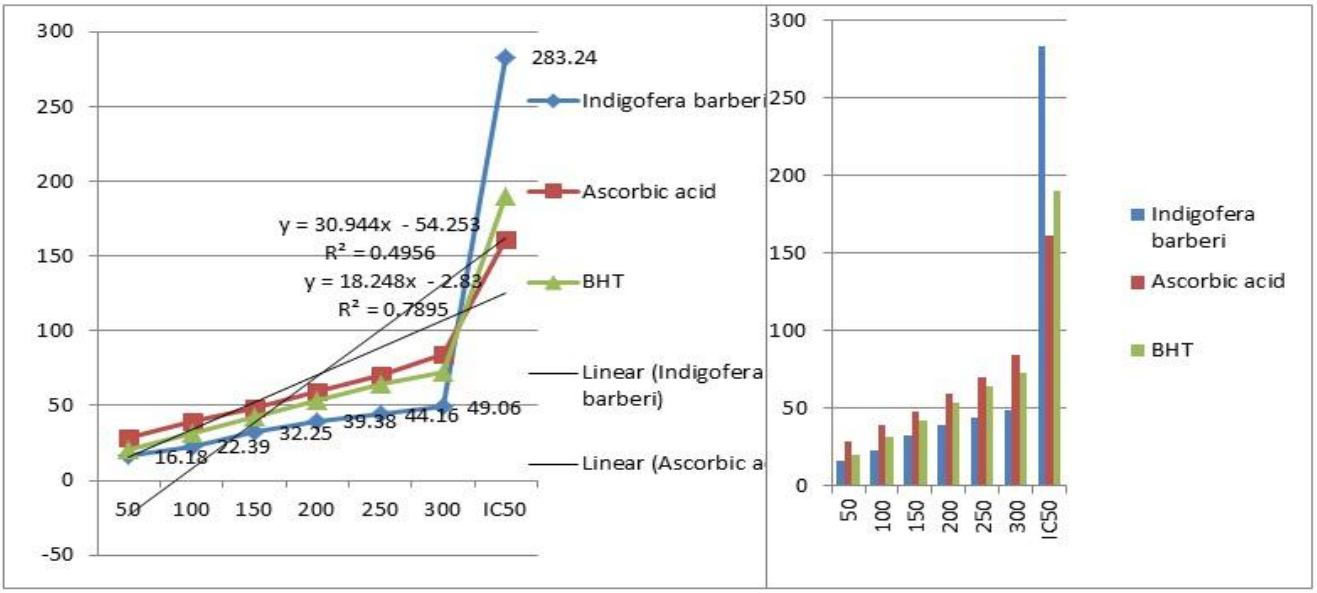


Figure.3 Graphical representation of free radical production by H_2O_2 with *IBEE*

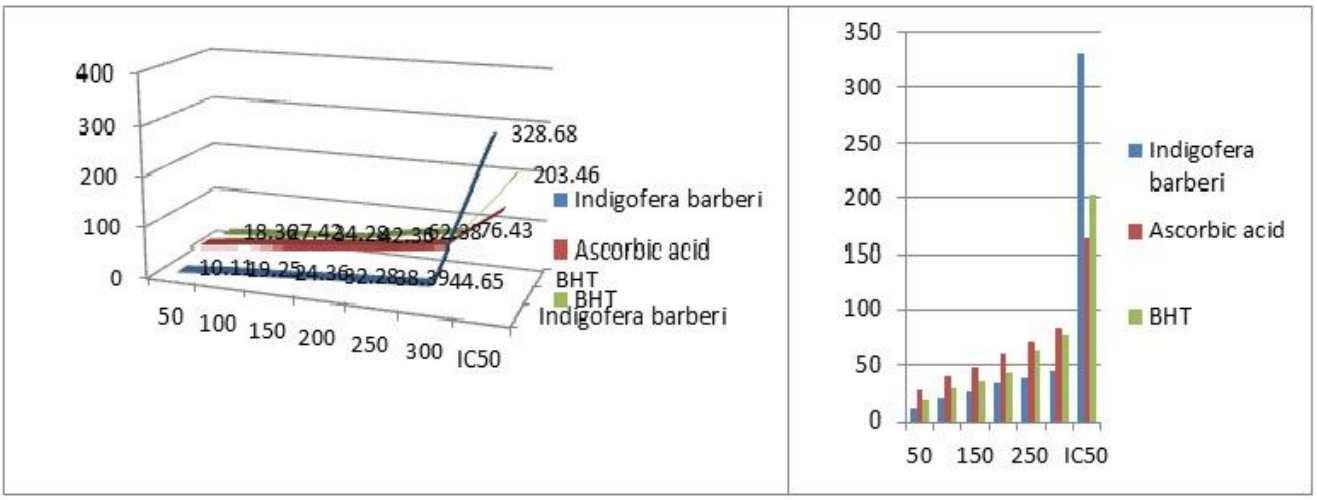


Figure.4. Graphical representation of metal chelating effect by70% *IBEE*

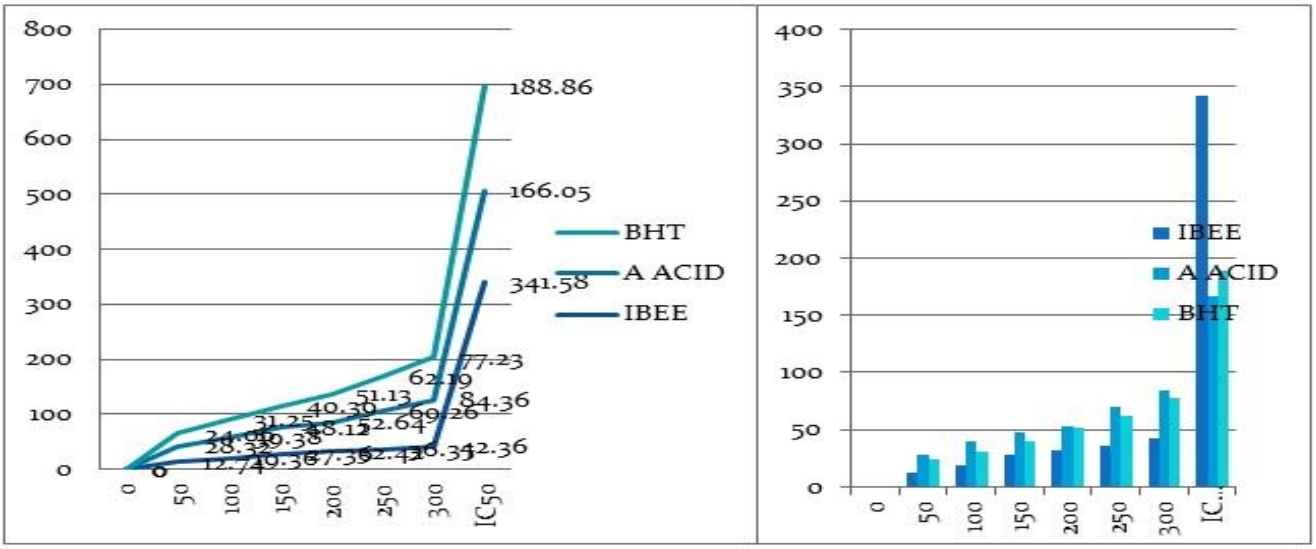
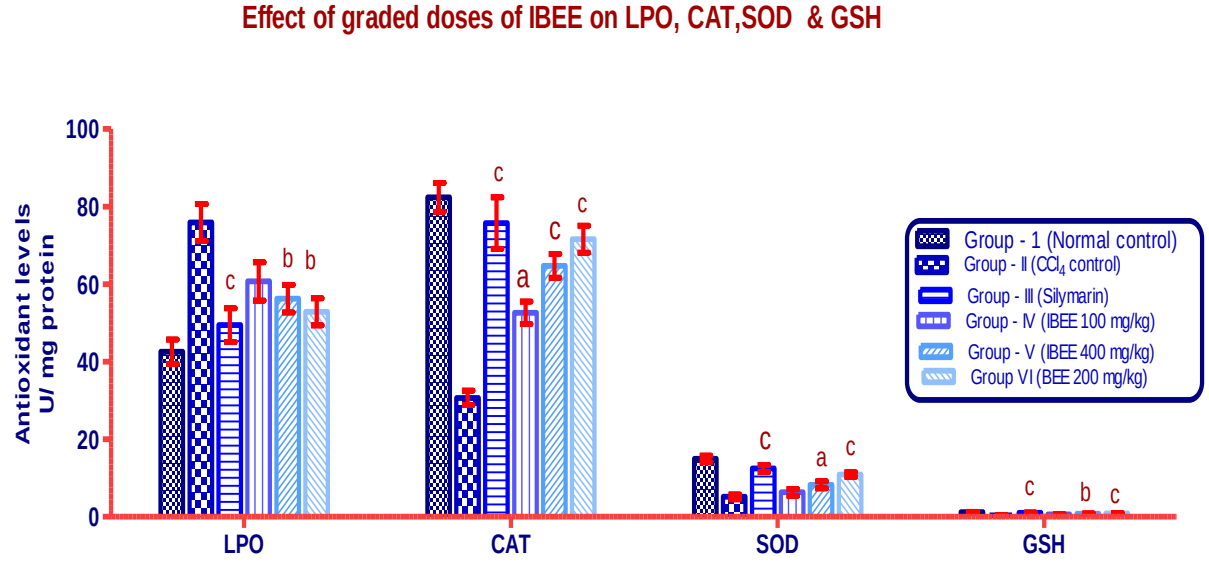


Figure.5 Graphical representation of IBEE on anti-oxidant enzymes in CCl_4 induced hepatotoxicity model



Data represent the Mean ± SEM, (n=6). Statistical significance: [a]P< 0.05, [b]P< 0.01, [c] P < 0.001, versus with respect to CCl_4control groups (One way ANNOVA followed by Dunnet's multiple comparison test)

Acute Toxicitity Studies (OECD – 423)

Selection of doses

The dose selection for the 70% ethanol extracts of *Indigofera barberii* was based on the acute toxicity study 423, which did not show any adverse effect following oral administration of doses up to 4000 mg/kg b. wt. Accordingly, experimental oral doses of 100, 200 and 400 mg/kg that equal to one-fortieth (1/40), one – twentieth (1/20)& one – tenth(1/10) of the maximum possible dose of the extract that did not cause mortalities in rats were selected. The results obtained from the toxicology studies of ethanolic extract of different extracts of bark of whole plant of *Indigofera barberi* were summarized in Table.3.

***In Vitro* Antioxidant Activity**

The antioxidant activity of the 70% IBEE on DPPH assay, NO assay, H_2O_2, Superoxide Anion Radical Scavenging Activity & metal chelating activity were determined.

DPPH *(1, 1-diphenyl-2-picrylhydrazyl)* method

The disappearance of DPPH was directly proportional to the antioxidant present in the reaction mixture (antioxidant react with stable free radical i.e. α,α-diphenyl-β-picrylhydrazyl (deep violet) and convert it to α,α-diphenyl-β-picrylhydrazine with discoloration). The highest % inhibition of *IBEE*, Ascorbic acid &*BHT* was found to be 45.28±1.84, 79.16±1.98&72.38±1.81 at 250µg/ml were recorded at a concentration of 250 µg/ml.The IC_{50} value of *IBEE* was determined to be 274.44µg/ml. The extracts in all concentrations showed the percentage of inhibition on free radicals and the actions were dose dependent. The percentage of scavenging effect on the DPPH was increased with increasing concentrations of ethanolic extract of *Indigofera barberi* (*IBEE*) were given in the Table.4 & Figure.1.

The % inhibition of NO by 70% /*IBEE* (B)

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions & were given in Table.5 & Figure.2. The effect of scavenging effect *IBEE* on the NO radical was increased with the increase in the concentrations of the extract from 50 -300 µg/ml. The percentage of inhibition of the *IBEE* on NO radical was varying from 16.18 ±0.46 to 49.06 ± 1.32µg/ml (in 50 µg/ml to300 µg/ml of extract). The IC_{50} value of *IBEE*, Ascorbic acid and BHT were determined to be 283.24, 161,33& 190.55 µg/ml. The extracts in all concentrations showed the percentage of inhibition on free radicals were concentration dependent.

The % inhibition of H_2O_2 by 70% *IBEE*

The antioxidant activity of the 70%*BOEE, IBEE & RBEE* on H_2O_2were determined and the results were given in Table.6 & Figure.3. H_2O_2 Scavenging activity of extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Thus, removal of H_2O_2 is very important for protection of food systems. The percentage of scavenging effect on H_2O_2 was increased with the increase in the concentrations of the extract from 50 – 300µg/ml. The percentage of inhibition of the H_2O_2 was varying from 12.16±0.67 to 52.35% ± 1.58 (in 50 –300µg/ml extract). The IC_{50} value of the ethanolic bark extract of the study species was 289.6µg/ml.

Metal chelating activity

The antioxidant activity of the 70% *BOEE, IBEE*&*RBEE* on Metal chelating activity were determined and the results were given in Table.11 & Fig.15. The percentage inhibition of *IBEE*, Ascorbic acid and BHT were concentration dependent and it was increasing with the increase in the concentration of extracts, Ascorbic acid and BHT from 50 to 300µg/ml. The percentage inhibition of*IBEE* on the metal chelation was varying from 12.74±0.68 to42.36± 2.81 (in50 – 300µg/ml). The IC50 value of *IBEE* was341.58 µg/ml. The percentage inhibition of *IBEE* in various concentrations, on metal chelating activity was less than the respective concentration of Ascorbic acid & BHT.

The I*BEE* extracts produced concentration dependent action. The dose of 300µg/ml of *BOEE, IBEE & RBEE* produced highest percentage inhibitory action on metal chelating activity then the other contraction of extracts. *RBEE* at the concentration of 300 µg/ml produced maximum % inhibitory then the *BOEE* and *IBEE* at 300 µg/ml.

***In Vivo* antioxidant studies**

Effect of graded doses of extract of *IBEE* on antioxidant parameters

The rats treated with CCl_4showed a marked effect on LPO, CAT, SOD & GSH levels when compared to normal control group. Treatment with at different doses of 100, 200 & 400 mg/kg b. wt of *BOEE, IBEE, RBEE*& 50 mg/kg b.wt of silymarin had showed significant effect on the LPO, CAT, SOD & GSH levels as compared to toxicant group, which was evident by in the Table.8 & Figure.5.

Effect *IBEE*

The effect of different doses of *IBEE* increased the decreased levels of CAT, SODand GSH& decreased the increased levels of LPO. The doses of 100,200 & 400 mg/kg b.wt of*IBEE* increased the decreased levels of antioxidants. 400 mg/kg b.wtt had showed the potent action than the 100 & 200 mg/kg b.wt of *IBEE*. The action were dose dependent, CAT were 41.68, 52.64, 47.19%, SOD: 18.48, 37.45, 52.66% GSH: 33.89, 51.85, 56.66%. 400 mg/kg b.wt of *BOEE, IBEE*&*RBEE* produced the increased levels of CAT; SOD & GSH were more effective than the 100 & 200 mg/kg b.wt of *BOEE*, IBEE & RBEE. At the dose of 400 mg/kg b.wt of *RBEE*

produced more protective effect than the 400 mg/kg b.wt of *BOEE* & *IBEE*.

DISCUSSION

Free radicals have been implicated in the causation of several problems like asthma, cancer, cardiovascular disease, cataract, diabetes, gastrointestinal inflammatory disease, liver disease, muscular degeneration and other inflammatory process (Sen S et al., 2010). Reactive oxygen species (ROS) are continuously produced during cell metabolism and under normal conditions; they are scavenged and converted to nonreactive species by different intracellular enzymatic and non-enzymatic antioxidant system (Shao HB et al., 2008). Over production or an ineffective elimination of ROS may induce oxidative stress and cause damage to all types of biomolecules such as proteins, lipids and nucleic acids (Droge W, 2002).

Antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules and or activators of a ntioxidative defense enzyme system to suppress the radical damages in biological systems (Murphy MP et al 2011; Venkatesh S et al, 2009). Antioxidants thus play an important role in the protection of human body against damage by reactive oxygen species (Peng KT et al, 2011; Ling Lu et al., 2011). Antioxidants that scavenge reactive oxygen species may be of great value in preventing the onset and/or the propagation of oxidative diseases (Willet WC, 1994). The most common antioxidants present in vegetables and part of the plants are vitamins C, vitamin E, Carotenoids, phenols and flavonoids. *In vitro* antioxidant activity of the ethanolic bark extract of Indigofera barberi was investigated in the present study by DPPH, nitric oxide scavenging activity, hydrogen peroxide scavenging activity, and metal chelating assays. The antioxidative effect is mainly due to phenolic components, such as phenolic acids, phenolic diterpenes and the presence of hydroxyl groups (Shahidi F et al., 1992). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa T, 1994). Phenolic compounds &terpenoids have been shown to be responsible for the antioxidant activity of plant extracts (Rice – Evans C et al., 1996). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH, transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to 1 - 1 diphenyl - 2 - picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug (Sochor J, 2010). The compound, SNP is known to decompose in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions NO. Reacts with oxygen to produce stable products (nitrate and nitrite). This leads to reduction of nitrite concentration in the acetic media (Ara N, Nur H, 2009). H2O2 is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells (Sakat SS et al., 2010). Reducing capacity of the extract components may serve as a significant indicator of its potential antioxidant activity (Meir S, 1995). The presence of transition metal ions in a biological system could catalyse the Haber-Weiss and Fenton type reactions, resulting in generation of hydroxyl radicals (OH). However, these transition metal ions could form chelates with the antioxidants, which results in the suppression of OH -generation and inhibition of peroxidation processes of biological molecules (Arulmozhi S et al., 2010). It may be due to the chelating agents, which form sigma bonds with the metal and effective as secondary antioxidants because they reduce the redox potential, thereby the oxidized form of the metal ion. In the present study, it is found that ethanolic extract of showed concentration dependent free radical scavenging activity and this antioxidant effect may be due to the higher content of alkaloids, flavonoids, steroids, glycosides, tannins and phenols (Chew YL et al., 1973). Thus, Indigofera barberi extract as promising natural sources of antioxidants can be used in nutritional or pharmaceutical fields for the prevention of free-radical-mediated diseases. A major defence mechanism involved in hepatotoxicity was in vivo antioxidant mechanism. The enzymatic antioxidant defence systems are the natural protector against lipid peroxidation. Administration of CCl_4 leads to generation of peroxy radical O_2, which is associated with inactivation of CAT, SOD & GSH enzymes and non enzymes (Zimmermann R et al., 1973; Cheeseman KH, 1995; Mates JM 1999). This probably explains the significantly reduced activities of CAT, SOD & GSH observed in rats treated with CCl_4. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage. A reduction in the activities of these enzymes was associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes. The CCl_4-treated rats except normal and negative control (Group 1 & II) were treated with the administration of 100, 200, 400 mg/kg of BOEE/IBEE/RBEE & 50 mg of silymarin produced the increased CAT, SOD activities and GSH content.

CONCLUSION

The antioxidant activity exerted by different concentration (50 - 300 µl/ml) of 70% ethanolic extract of Indigofera barberi exhibited maximum percentage inhibition of DPPH, NO, H_2O_2 and metal chelating activity, the actions were concentration dependent. The result of the present study showed that the plants extract such as 70% ethanolic extract of whole plant of Indigofera barberi IBEE which contains phenolic and flavonoidal compounds, exhibited the great antioxidant mechanism. The high scavenging property of these extract may be due to hydroxyl groups existing in the phenolic compounds' that can provide the necessary component as a radical scavenger. Further research investigations may be carried out to isolate the actual phyto constituents responsible for antioxidant activity.

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