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ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITY OF SAPONINS OF *ALBIZZIA LEBBECK* BARK AND LEAVE

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Abstract

This study was designed to determine the antioxidant and hepatoprotective effect of *n*- butanol soluble fractions of *Albizzia lebbeck* bark and leaf extract. The various extracts were prepared and extracts containing flavanoids, tannins and saponins (Methanolic extract) was selected for isolation of chemical constituents. *n*- butanol soluble fractions of *Albizzia Lebbeck* bark extract (BFALB) and leaf extract (BFALL) were selected for screening of *in-vitro* antioxidant activity such as reducing power, superoxide anion and hydroxyl radical scavenging activity. Both fractions were subjected to acute toxicity study. BFALB and BFALL were also screened for its influence on tissue GSH levels and lipid peroxidation (*In-vivo* antioxidant models). The 60 mg/Kg BFALB and 20 mg/Kg BFALL were further screened for hepatoprotective (against CCl_4 induced hepatotoxicity) activities. The various relevant biochemical markers like SGOT, SGPT, ALP, Total Bilirubin, Direct Bilirubin were estimated to assess the hepatoprotective potential of the fractions. BFALB and BFALL demonstrated dose dependant *in vitro* & *in vivo* antioxidant activities in all the models of the study. The treatments with fractions have reversed all the biochemical markers of hepatotoxicity to the near normal levels. From the results it may be concluded that the *n*-butanol soluble fractions of *Albizzia lebbeck* bark and leaf possess antioxidant and hepatoprotective activity. The antioxidant and hepatoprotective activity of the plant may be attributed to the polyphenolic compounds like saponins that are present in the plant.

INTRODUCTION:

The human beings are exposed to environmental, occupational and xenobiotics challenges due to modern life style. Enormous free radicals are generated during the exposure to such stressful challenges. The continuous exposure to the stressful conditions may be overpowering the inbuilt scavenging capacity of the cells leading to cellular destruction. The various free radicals that are released into the body are superoxide anion (O_2•), NO radical, $NOO^{\bullet}$, $OH^{\bullet}$ and H_2O_2 radical. Thus released free radicals react with the membrane polyunsaturated lipids and oxidize them to lipid peroxides. This lipid peroxidation damage membrane protein as well as the lipids. Thereby the integrity of membrane is lost. Therefore it is considered that the extent of lipid peroxidation is directly proportional to cell damage. In addition the free radicals may also attack DNA and causes tissue damage.

Therefore several attempts have been made to prevent and treat hepatotoxicity by using several antioxidants principles. Most of these antioxidants are from natural origin. Herbs are the major source of antioxidants. Basically phytoconstituents like (quercetin, β-carotene, tocopherol), tannins, vit-C, vit-E are major antioxidants principles. Many herbs containing these type phytoconstituents have been investigated for their antioxidants and organprotective properties and reported that many of such plants are useful as organ protectants. In one of our field survey we found a tree by name *Albizzia lebbeck (*Linn.*).* Upon literature survey it was found that enormous research has been carried out on the phytochemical profile of the plant. And there are reports that saponins-labbeckanin C, labbecknin A, labbecknin E, d-catechin, d- leucocyanidin, β-sitosterol, melacacidin , friedelan-3-one, flavon., and tannins, etc. have been isolated from the plant.

The polyphenolic compounds like flavanoids, tannins and saponins possess anti-oxidant property [1-5]. Similarly there are reports that anti-oxidants have a role in hepatoprotective, activity [6, 7]. So in the present study, the isolated phytoconstituents of bark and leaf extracts were taken for assessing the antioxidant and hepatoprotective activity.

MATERIALS AND METHODS:

Plant Material:

Albizzia lebbeck bark and leaf were collected from Sarsa, near Sarsa Cross road, Gujarat in the month of December. The plant was authenticated by Dr. G. C. Jadeja, Prof. and Head of Botany, Department of Agricultural Botany, B.A. College of Agriculture, Anand Agriculture University, Anand. A herbarium specimen (Skcop-2010-1) is deposited in the college herbal museum for future reference.

Isolation of saponins:

The bark and leaf were shade dried separately at room temperature and pulverized. The powdered plant material (1 kg) was sequentially extracted three times with 3 L of petroleum ether, chloroform, methanol and water at room temperature for 48h in a soxhlet apparatus. All the extracts were concentrated by distilling the solvents and the extracts were dried in an oven at 40^0C. Each time before extracting with the next solvent, the marc was dried in an air oven below at 40^0C.The marc was finally macerated with water for 24 hours to obtain the aqueous extract. The completion of the extraction was confirmed by evaporating a few drops of extract from the thimble on watch glass to observe that no residue remained after evaporation of the solvent. The extracts obtained with different solvents were collected and stored in a refrigerator. As described by Pal et al [8], the residue was suspended in water, extracted with ethyl acetate and *n*-butanol (3 x 300 ml each) and the solution was evaporated to dryness in vacuum. The *n*-butanol soluble fraction of *Albizzia Lebbeck* bark extract (BFALB) and *n*-butanol soluble fraction of *Albizzia Lebbeck* leave extract (BFALL) were tested for the presence of saponins using haemolysis test and foam test as described earlier by Evans (1996)[9].

Animal:

Albino rats (Wistar) weighing 150-200g and albino mice weighing 20-25g of either sex were used in this study. They were procured from Flair Labs, Palsana, Surat. The animals were acclimatized for one week under laboratory conditions. They were housed in polypropylene cages and maintained at 27°C ± 2°C under 12 hrs dark / light cycle. They were fed with standard rat feed (VRK Nutritional Solutions) and water *ad libitum* was provided. The husk in the cages was renewed thrice a week to ensure hygeinity and maximum comfort for animals. Ethical clearance for handling the

animals was obtained from the Institutional animal ethical committee prior to the beginning of the project work (Protocol No: IAEC/SKCOP/11-12/02).

Acute Toxicity studies:

The acute toxicity for *n*-butanol soluble fraction of *Albizzia Lebbeck* bark extract (BFALB) and *n*-butanol soluble fraction of *Albizzia Lebbeck* leave extract (BFALL) were determined on albino mice, maintained under standard conditions. The animals were fasted overnight prior to the experiment. Fixed dose method of OCED Guideline No.425 was adopted for toxicity studies [10].

Reducing power:

The reducing power of BFALB and BFALL were determined according to the method of Oyaizu[11]. Different doses of BFALB and BFALL were mixed in 1 ml of distilled water so as to get 20µg, 40µg, 60µg, 80µg and 100µg concentration. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700nm. Increased absorbance of the reaction mixture indicates increase in reducing power.

The % reducing power was calculated by using the formula:

$$\%\ increase\ in\ absorbance = \frac{Test\ OD - Control\ OD}{Control\ OD}\ x\ 100$$

Superoxide anion scavenging activity:

Measurement of superoxide anion scavenging activity of BFALB and BFALL were done by using the method explained by Nishimiki [12] and modified by Ilham et al [13]. About 1 ml of nitroblue tetrazolium (NBT) solution (156µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468µM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of BFALB, BFALL and standard in water were mixed. The reaction was started by adding 100µl of Phenazine methosulphate (PMS) solution (60µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 minutes, and the

absorbance at 560 nm was measured against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. % inhibition of OD was calculated by using the formula.

$$\%\ inhibition = \frac{Control\ OD - Test\ OD}{Control\ OD}\ x\ 100$$

Hydroxyl radical scavenging activity:

Hydroxyl radical generation by phenyl hydrazine has been measured by the 2-deoxyribose degradation, assay of Halliwell and Gutteridge [14]. In 50mM phosphate buffer (pH 7.4), 1 mM deoxyribose, 0.2 mM phenyl hydrazine hydrochloride were prepared. 0.6ml of 1mM deoxyribose and 0.4ml of BFALB, BFALL and standard were taken. 0.2 ml phosphate buffer was added to make reaction solution 1.6ml. After 10 min incubation 0.4ml of 0.2 mM phenyl hydrazine was added. Incubation was terminated after 4 hrs and 1 ml each of 2.8% TCA and 1 % (w/v) thiobarbituric acid were added to the reaction mixture and heated for 10 mins in a boiling water bath. The tubes were cooled and absorbance was taken at 532 nm. Decrease in absorbance is indicating the increase in the hydroxyl free radical scavenging activity. The % inhibition in the OD is calculated by using the formula mentioned earlier. **CCl_4 induced hepatotoxicity:**

The method of Suja SR. et al [15]. was followed. In the dose response experiment, albino rats were randomly assigned into five groups of six individuals each. Group-I and Group II received distilled water (1ml/ kg) for 5 days. Group III received 100 mg/kg silymarin (standard drug) orally for 5 days. Group IV and Group V received 60 mg/kg BFALB and 20 mg /kg BFALL (orally) respectively for 5 days. Group-I received liquid paraffin (1ml/kg) s.c., on 2nd and 3rd day. Group-II, III, IV and V received CCl_4: liquid paraffin (1:1) at a dose of 2ml/kg s.c., on 2nd and 3rd day, after 30 min of vehicle, 100 mg/kg silymarin, 60 mg/kg BFALB and 20 mg/kg BFALL administration. Animals were sacrificed on the 6th day under mild ether anesthesia. Blood samples were collected for evaluating the serum biochemical parameters like SGOT, SGPT, ALP, Total Bilirubin and Direct Bilirubin. The liver samples were dissected out, blotted off blood, washed with saline and used to estimate the liver weight, liver volume, GSH estimation and LPO estimation.

Biochemical studies:

Blood was obtained from all the animals by puncturing retro-orbital plexus. Collected blood was centrifuged (2000 rpm for 10 mins) to get clear serum and was used to estimate various biochemical markers like SGPT[16], SGOT[17], ALP[18], Bilirubin(total and direct)[19].

In vivo tissue GSH estimation:

Tissue samples (liver) were homogenized in ice cold Trichloroacetic acid (1 gm tissue plus 10 ml 10% TCA) in an ultra turrax tissue homogenizer. Glutathione measurements were performed using a modification of the Ellamn procedure [20]. Briefly, after centrifugation at 3000 rpm for 10 minutes, 0.5 ml supernatant was added to 2 ml of 0.3 M disodium hydrogen phosphate solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml in 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. % increase in OD is directly proportional to the increase in the levels of Glutathione. Hence, % increase in OD is calculated.

In vivo lipid peroxidation estimation:

Lipid peroxidation, is accepted to be one of the principal causes of CCl_4-induced liver injury, and is mediated by the production of free radical derivatives of CCl_4 [21]. The degree of lipid peroxide formation was assayed by monitoring thiobarbituric reactive substance formation [22,23]. Stock solution of TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25N hydrochloric acid. This solution may be mildly heated to assist in the dissolution of the thiobarbituric acid. Combine 1.0 ml of biological sample (0.1-2.0 mg of membrane protein or 0.1-0.2 µmol of lipid phosphate) with 2.0 ml of TCA-TBA-HCl and mix thoroughly. The solution is heated for 1 hr in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 2 min. The absorbance of the sample is determined at 535 nm against a blank that contains all the reagents minus the lipid. The malondialdehyde concentration of the sample can be calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Statistical analysis:

Results were expressed as mean ± SEM, (n=6). Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test by using Graph Pad Instat

Software. P value less than 0.05 was considered to be statistically significant.

RESULTS:

Acute toxicity:

The mice treated with the 40 and 60 mg/kg of BFALB and 10 and 20 mg/kg of BFALL orally, exhibited normal behavior, i.e. they were alert, with normal grooming, touch response, and pain response. There was no sign of passivity, stereotypy, and vocalization. Their motor activity and secretory signs were also normal. In the Animals treated with 300 mg/kg of BFALB and 100 mg/kg of BFALL, 50% of animal could not survive. Therefore 60 mg/kg of BFALB and 20 mg/kg of BFALL was selected for further study i.e. for screening of hepatoprotective activity.

Antioxidant activity:

In all models, BFALB and BFALL showed concentration dependent antioxidant activity (Table No 1). The BFALB showed 125.68% reducing power, 76.78% super oxide anion and 43.25% hydroxyl radical scavenging activities, whereas BFALL showed 135.95% reducing power, 80.13% superoxide anion and 51.52% hydroxyl radical scavenging activities at 100 mcg concentration which are comparable to that of Sodium metabisulfate 25 mcg.

In vivo GSH:

There was a marked depletion of GSH level in CCl_4 treated group. Silymarin 100 mg/kg increased tissue GSH by 94.32%. Treatment with BFALB and BFALL showed increase in the levels of GSH. However, BFALB and BFALL have shown lesser increase GSH level than standard silymarin (Table No 2).

In vivo lipid per oxidation:

CCl_4 has enhanced the lipid peroxidation. The treatment with BFALB and BFALL has significantly reduced the lipid per-oxidation. Silymarin 100 mg/kg showed 55.67% inhibition, whereas 20 mg/kg of BFALL showed 53.19% inhibition, which was almost near to standard silymarin (Table No 2).

CCl_4-induced hepatotoxicity:

Increased levels of liver weight (4.87gm/100gm), liver volume (5.18 ml/100gm), SGPT (335.75IU/L), SGOT (401.01IU/L), ALP (452.33IU/L), total bilirubin (3.39 mg/dl) and direct bilirubin (1.72 mg/dl) observed in CCl_4 treated group. The pretreatment with BFALB and BFALL (60 mg/kg and 20 mg/kg p.o. respectively)

has brought back the elevated levels of biomarker enzymes of hepatotoxicity (Table No 3). Treatment with 20 mg/kg of BFALL has produced the hepatoprotective activity comparable to that of silymarin 100 mg/kg p.o.

DISCUSSION:

CCl_4 is metabolized to trichloromethyl ($CCl_3^{\bullet}$) radical which is further converted to trichloromethyl peroxy radical by superoxide anions. This trichloro methyl peroxy radical is the main culprit in causing hepatotoxicity. This particular radical forms a covalent bond with sulphydryl group of membrane GSH, protein thiols and unsaturated fats or lipids. This covalent bonding of free radicals with cellular macro molecules initiates the cascade of reactions leadings to lipid peroxidation [24-27]. The lipid peroxidation inturn alter the membrane permeability and initiates chain of reaction leading to tissue damage and necrosis.

It was observed that the both the *n*-butanolic fractions showed significant reducing power and superoxide anion scavenging activity. Therefore pretreatment with BFALB and BFALL may be preventing the formation of trichloro methyl peroxy radical due to superoxide anion scavenging activity. Thereby tissue GSH levels are not depleted and lipid peroxidation is minimized, this may be the possible mechanism of hepatoprotection offered by BFALB and BFALL. However our study does not confirm whether text extract block CYP 450 $2E_1$ enzyme and thereby inhibit the formation trichloromethyl $CCl_3^{\bullet}$ radical. The results are inconformity with the earlier reports.

CONCLUSION:

In conclusion, the present study demonstrates that BFALB and BFALL possess antioxidant and hepatoprotective activity. In addition, the hepatoprotective property may be attributed to the antioxidant principles of plant, namely saponins, tannins and flavanoids. Further investigation is going on to isolate, characterize and screen the active principles that possess antioxidant and hepatoprotective property.

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facilities to carry out this research work. We are also thankful to Microlabs, Bangalore for providing standard drug (Silymarin).

Table No. 1: *In vitro* antioxidant activity of BFALB and BFALL

Treatment	Reducing power Mean ± SEM (% increase)	Superoxide anion Scavenging Mean ± SEM (% inhibition)	Hydroxyl radical scavenging Mean ± SEM (% inhibition)
Control	0.292 ± 0.0023	0.448 ± 0.0033	0.326± 0.0005
Control + standard 25 µg	0.570 ± 0.0017*** (95.20)	0.031 ± 0.0030*** (63.44)	0.114 ± 0.0015*** (65.03)
Control + BFALB 20 µg	0.358 ± 0.0028*** (22.60)	0.368 ± 0.0026*** (10.75)	0.269 ± 0.0056*** (17.48)
Control + BFALB 40 µg	0.423 ± 0.0028*** (44.86)	0.245 ± 0.0019*** (17.56)	0.248 ± 0.0024*** (23.92)
Control + BFALB 60 µg	0.489 ± 0.0037*** (67.46)	0.225 ± 0.0025*** (21.16)	0.237 ± 0.0041*** (27.30)
Control + BFALB 80 µg	0.598 ± 0.0003*** (104.69)	0.149 ± 0.0018*** (24.37)	0.212 ± 0.0056*** (34.96)
Control + BFALB 100 µg	0.659 ± 0.0014*** (125.68)	0.104 ± 0.0036*** (41.21)	0.185 ± 0.0032*** (43.25)
Control + BFALL 20 µg	0.382 ± 0.0020*** (30.82)	0.342 ± 0.0039*** (15.17)	0.258 ± 0.0046*** (20.85)
Control + BFALL 40 µg	0.454 ± 0.0018*** (55.47)	0.232 ± 0.0018*** (19.35)	0.239 ± 0.0038*** (26.68)
Control + BFALL 60 µg	0.552 ± 0.0029*** (89.04)	0.201 ± 0.0027*** (27.59)	0.225 ± 0.0032*** (30.98)
Control + BFALL 80 µg	0.643 ± 0.0010*** (120.20)	0.125 ± 0.0029*** (38.35)	0.191 ± 0.0048*** (41.41)
Control + BFALL 100 µg	0.689 ± 0.0019*** (135.95)	0.089 ± 0.0009*** (46.59)	0.159 ± 0.0028*** (51.52)

Values are the mean ± S.E.M., n=3

Significance *** P<0.001 compared to control.

STD: Sodium metabisulphate

Table No. 2: Effect of BFALB and BFALL on tissue GSH and Lipid peroxidation levels in CCl_4 induced hepatotoxicity

Treatment	Tissue GSH level		Tissue lipid peroxidation	
	Mean ± SEM	**% Increase**	**Mean ± SEM**	**% Inhibition**
Normal Control (1ml vehicle)	0.982 ± 0.031	--	0.203 ± 0.027	--
CCl_4 Control CCl_4 + Liq. Paraffin (1:1) (2 ml/kg s.c.)	0.476 ± 0.034	--	0.485 ± 0.028	--
CCl_4 + Silymarin (2 ml/kg s.c. + 100 mg/kg p.o.)	0.925 ± 0.045***	94.32%	0.215 ± 0.023***	55.67%
CCl_4 + BFALB (2 ml/kg s.c. + 60 mg/kg p.o.)	0.798 ± 0.019***	67.64%	0.241 ± 0.015***	50.30%
CCl_4 + BFALL (2 ml/kg s.c. + 20 mg/kg p.o.)	0.869 ± 0.030***	82.56%	0.227 ± 0.028***	53.19%

Values are the mean ± S.E.M., n=3

Significance ***P<0.001 compared to CCl_4 treatment.

Table No. 3: Effects of BFALB and BFALL on Liver weight, liver volume and biochemical markers in CCl_4 induced hepatotoxicity

Treatment	Liver		Biochemical parameters				
	Volume (ml/100g)	**Weight (g/100g)**	**SGOT IU/L**	**SGPT IU/L**	**ALP IU/L**	**Total Bilirubin mg/dl**	**Direct Bilirubin mg/dl**
Normal Control (1ml vehicle)	3.57 ± 0.12	3.15 ± 0.10	132.23 ± 6.76	55.72 ± 2.74	139.21 ± 3.57	0.83 ± 0.02	0.16 ± 0.04
CCl_4 Control	5.18 ± 0.27	4.87 ± 0.26	401.01 ± 7.34	335.75 ± 10.93	452.33 ± 9.61	3.39 ± 0.05	1.72 ± 0.06
CCl_4 + Liq. Paraffin (1:1) (2 ml/kg s.c.)							
CCl_4 + Silymarin (2 ml/kg s.c. + 100 mg/kg p.o.)	3.69 ± 0.17***	3.38 ± 0.13**	163.82 ± 6.34***	59.43 ± 8.71***	191.54 ± 6.82***	1.10 ± 0.04***	0.28 ± 0.02***
CCl_4 + BFALB (2 ml/kg s.c. + 60 mg/kg p.o.)	3.81 ± 0.23***	3.62 ± 0.30**	192.71 ± 8.49***	71.94 ± 5.48***	249.48 ± 8.56***	1.97 ± 0.04**	1.09 ± 0.03***
CCl_4 + BFALL (2 ml/kg s.c. + 20 mg/kg p.o.)	3.62 ± 0.19***	3.49 ± 0.16***	172.63 ± 7.74***	64.04 ± 7.92***	201.62 ± 7.78***	1.25 ± 0.03***	0.41 ± 0.05***

Values are the mean ± S.E.M. of six rats/ treatment

Significance **P <0.01 and *** P<0.001, compared to CCl_4 treatment

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