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Assessment of the antioxidant activity of aqueous extract of *Acacia catechu* bark: An *in vitro* and *in vivo* study

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ABSTRACT

Safer antioxidants are essential to prevent the progression of free radical mediated disorders. Plants present a large source of natural antioxidants that might serve as leads for the development of novel drugs. The present study investigates the antioxidant properties of aqueous extract of bark of *Acacia catechu in vitro* and *in vivo*. The *in vitro* tests in diversified fields included total antioxidant activity, scavenging activities for DPPH and H_2O_2, reducing power and phenolic content. The IC_{50} values for DPPH and H_2O_2 scavenging were found to be 177.53 ± 12.35 µg/ml and 455.2 ± 15.48 µg/ml respectively. The plant extract reduced the most Fe^{3+} ions in reducing power estimation. The extract yielded 67.40 ± 0.279 mg/ml gallic acid equivalent phenolic content per 100 mg plant extract. In the *in vivo* experiments, the extract treatment showed significant increase in the level of superoxide dismutase, catalase, glutathione-S-transferase and reduced glutathione at the dose of 100 and 200mg/kg bwt. The extract showed no toxicity, as the levels of the toxicity marker enzymes were found to be in the normal range. The present study provides evidence that aqueous extract of *Acacia catechu* bark shows potential antioxidant and free radical scavenging activity which might be due to the presence of phenolic compounds.

Keywords: Antioxidant, Phenolic content, Reducing power, Free radical scavenging.

INTRODUCTION

Disturbance of the balance between the production of reactive oxygen species (ROS) such as superoxide; hydrogen peroxide; hypochlorous acid; hydroxyl, alkoxyl, and peroxyl radicals; and antioxidant defenses against them produces oxidative stress. Oxidative stress can cause cellular damage and subsequent cell death because the reactive oxygen species oxidize vital cellular components such as lipids, proteins and DNA. The antioxidant system of body can reduce oxidative stress by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers.[1] But excessive generation of ROS, beyond the capacity of antioxidant system to control them, leads to the development of many diseases such as cancer, diabetes, liver injury, atherosclerosis, neurodegenerative disorders and cardiovascular diseases.[2-4] A potent scavenger of these species termed as antioxidant may serve as a possible preventive intervention for free radical-mediated diseases. There are two basic categories of antioxidant namely synthetic and natural ones. Restriction on the use of synthetic anti-oxidants such as butylated hydroxyanisole, butylated hydroxyltoluene and tertbutylhydroquinone is being imposed due to their carcinogenicity and side effects.[5] Thus the wide use of natural antioxidants as a replacement of synthetic antioxidants in food and food supplements has been employed owing to the fact that natural products are considered to be promising and safe source.

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Acacia catechu, a moderate size deciduous tree with dark grayish or brown rough bark, from the family leguminosae, is used extensively in South East Asian countries for preparing betel leaf (paan).[6] The dried bark of *Acacia catechu* commonly known as Katha or Karangali is widely used in India for its various pharmacological effects. Its use as an astringent and anti-inflammatory,[7] anti-bacterial and anti-fungal [8] hypoglycaemic and hepatoprotective [9] agent has been reported. Its use for treating high blood pressure, leucorrhoea, diarrhea, dysentery, leprosy, colitis, gastritis, bronchitis and cough, and also gargled for gingivitis, toothache, sore throat and mouth infections has also been reported. Hazra et al has reported the antioxidant and DNA protective properties of 70% methanolic extract of heartwood of *Acacia catechu*.[10] To the best of our knowledge, no study on the antioxidant properties of aqueous extract of the *Acacia catechu* bark has so far been reported so the present study was designed to evaluate the antioxidant properties of the aqueous extract of bark of *Acacia catechu* both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals:

Dithiobis 2-nitro benzoic acid (DTNB), sulfosalycilic acid, 1-chloro2,4-dinitro benzene (CDNB), ethylene diamine tetraacetic acid (EDTA), bismuth nitrate were obtained from Sisco Research Laboratories Pvt. Ltd, Delhi, India. Folin Ciocalteau, TCA, gallic acid, ninhydrin, α-naphthol, and vanillin were procured from Merck, Delhi, India. 1,1-diphenyl-2-picrylhydrazyl (DPPH), NADPH, oxidized glutathione, gallic acid, anisaldehyde were purchased from Sigma Chemicals, St.

Louis, MO, USA. The remaining chemicals and solvents used were of standard analytical grade and HPLC grade respectively.

Extract Preparation:

The bark of *Acacia catechu* was purchased from a local nursery and authenticated by National Institute of Science Communication and Information Resources (NISCAIR), Delhi, India (Voucher no.NISCAIR/RHMD/Consult/06/772/89). The dried bark was crushed to powder and soaked in water at room temperature. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized, and the resulting powder extract was used in the present study.

Estimation of antioxidant activity *in vitro*

Total antioxidant activity:

This activity was determined using method given by Prieto et al. (1999).[11] The assay is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH.

DPPH radical scavenging assay:

The free radical scavenging activity of the extract was evaluated by 1,1-diphenyl- 2-picrylhydrazyl (DPPH) using a standard method.[12] The disappearance of the free radical can be tracked by absorbance measurement at λ517nm. Briefly the reaction mixture (1.10 ml) contained 100 µl of aqueous extract or ascorbic acid and 1.00 ml of DPPH solution (0.1 mM in methanol). The control contained all the reaction reagents except test material. The reaction mixture was shaken well and allowed to react for 20 min at room temperature. The remaining DPPH free radical was determined by absorbance measurement at λ517 nm against methanol blanks. The percentage scavenging effect was calculated from the decrease in absorbance against control (without added test material).

Hydrogen peroxide scavenging assay:

This activity was determined by the method described by Ruch et al. (1989).[13] Briefly 1 ml of extract (prepared in phosphate buffered saline) was incubated with 0.6 ml of 4mM H_2O_2 solution (prepared in PBS) for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without H_2O_2.

Reducing Power assay:

This was determined according to the method of Oyaizu (1986).[14] Different concentrations of the aqueous extract of *Acacia catechu* (50 to 500 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%, 2.5 ml) was added to the mixture. A portion of the resulting mixture was mixed with $FeCl_3$ (0.1%, 0.5 ml) and the absorbance was measured at 700 nm in a spectrophotometer.

Estimation of phenolic content:

The phenolic content of the extract was determined according to Folin Ciocalteau method.[15] Briefly extract was dissolved in 25 ml of methanol, mixed by using vortex and 2 ml of this solution was added to 3 ml of 0.3% HCl. A 0.1 ml aliquot of the resulting solution was added to 2 ml of 2% Na_2CO_3 and after 2 min, 0.1 ml of Folin Ciocalteau reagent (diluted with methanol 1:1) was added. The reaction mixtures were then incubated in the dark at 25°C for 30 min and then the absorbance was measured at 750 nm. The concentration was calculated using gallic acid as standard and the results were expressed as milligram gallic acid equivalent per gram extract.

Phytochemical Analysis:

The chemical constituents of the aqueous extract were identified by qualitative chemical tests using the procedures of Kokate et al. (1996).[16]

Estimation of Antioxidant activity and toxicity *in vivo*

Experimental animals and treatments:

Healthy Wistar rats (weighing 180–230 g) were used for the study. The animals were housed in standard conditions of temperature (21°C ± 2°C), humidity (55±10%) and a 12 h light–dark cycle. The rats were fed with commercial diet and water *ad libitum*. For experimental purposes the animals were kept on fasting overnight but were allowed free access to water.

In order to assess the effect of aqueous extract of bark of *Acacia catechu* in normal healthy rats, four different doses were chosen viz., 50, 100, 150 and 200 mg/kg bwt. The experiment was carried out for one month with dose given orally once a day to five groups having six rats in each group (i) Group I: Control rats received vehicle as saline.(ii) Group II: Normal rats received 50 mg/kg bwt of the aqueous extract (iii) Group III: Normal rats received 100 mg/kg bwt of the aqueous extract (iv) Group IV: Normal rats received 150 mg/kg bwt of the aqueous extract (v) Group V: Normal rats received 200 mg/kg bwt of the aqueous extract.

Assay of different antioxidants:

After the last dose rats were dissected to remove their liver tissue, which was washed in ice cold saline (0.85% NaCl). The extraneous material was removed. The 20% homogenate of brain tissue in 0.1 M potassium phosphate buffer (pH 7.4) with 0.25 M sucrose was centrifuged at 800g for 5 min at 4°C in an IEC-20 refrigerated centrifuge (Rotar no. 894) to separate the nuclear debris. The supernatant obtained was centrifuged at 10,500g for 20 min at 4°C to obtain the post mitochondrial supernatant (PMS) which was used as a source for estimation of all the antioxidants. Reduced glutathione was measure by following the method of Jollow et al. (1974).[17] Glutathione-S-transferease activity was estimated according to the method of Habig et al. (1974).[18] Superoxide dismutase activity was measured using the procedure of Poaletti and Mocali (1990).[19] Catalase activity was assayed using the method of Cohen et al. (1970).[20] The protein content was measured by the method of Lowry et al. (1951).[21]

For toxicity analysis, blood was centrifuged at 1480×g for 10 min to obtain serum, which was stored at -20°C until determination of the following parameters according to standard assay kits: blood urea

nitrogen (BUN), creatinine, Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and creatinine.

Statistical analysis:

Results of the study are expressed as mean±S.E.M. Comparisons among the groups were done by one-way ANOVA using Graph Pad Prism, Version 4.0 (Graph Pad Software, San Diego, CA, USA). When the *p*-value obtained from ANOVA was significant ($p < 0.05$), the Tukey test was applied to test for differences among groups.

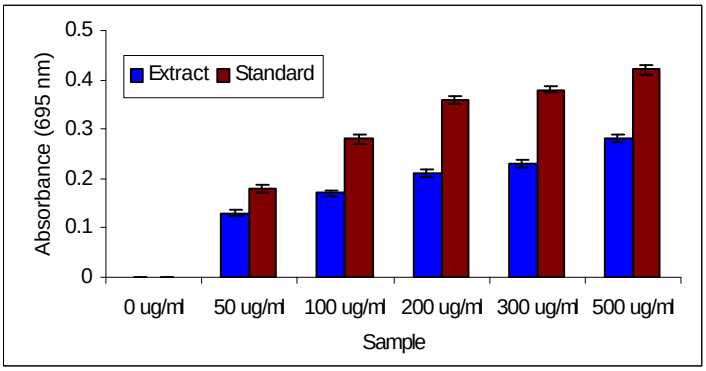
RESULTS

In vitro antioxidant activity

Total antioxidant assay

The total antioxidant activity of aqueous extract of the bark of *Acacia catechu* was calculated from the formation of a green coloured complex of phosphate and Mo(IV), which was measured spectrophotometrically at 695 nm. The activity was compared among different doses of extract as well as with the standard ascorbic acid. The high absorbance values indicated that the sample possessed significant antioxidant activity. The activity of extract at the concentration of 500µg was comparable with the standard. (Fig. 1.)

Fig. 1: Total antioxidant activity of *Acacia catechu* bark extract and the reference compound ascorbic acid.

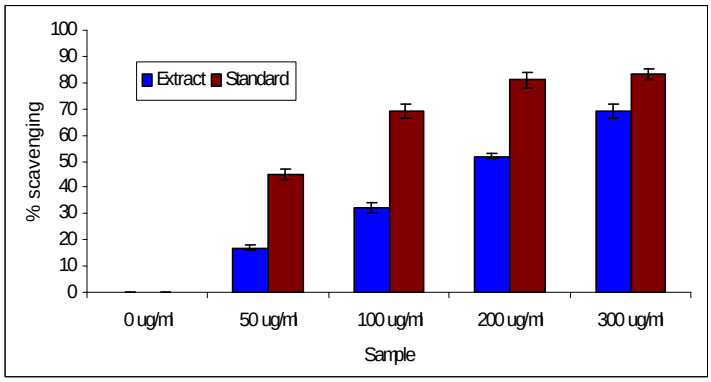


Each value represents mean ± S.E.M. (n=6)

DPPH free radical scavenging

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 colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. Comparison of the antioxidant activity of the extract and ascorbic acid is shown in Fig. 2. The aqueous extract of bark of *Acacia catechu* exhibited a significant dose dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 177.53 ± 12.35 µg/mL. The IC_{50} value of the extract was found to be comparable to reference standard ascorbic acid.

Fig. 2: DPPH radical scavenging activity of *Acacia catechu* bark extract and the reference compound ascorbic acid.

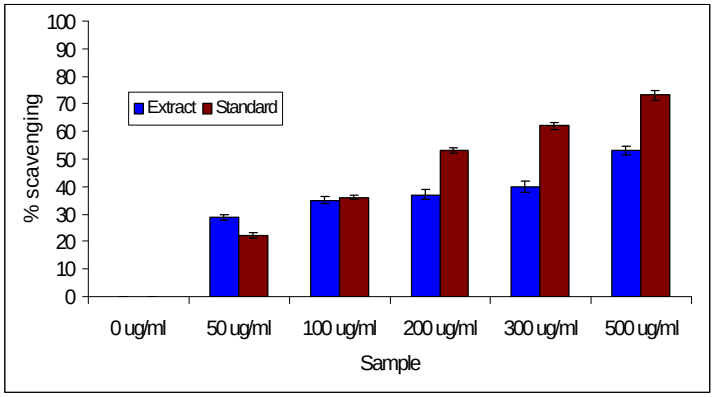


Each value represents mean ± S.E.M. (n=6)

H_2O_2 scavenging assay

The scavenging ability of species of *Acacia catechu bark* extract with H_2O_2 is compared with the ascorbic acid and is depicted in Fig. 3. Though H_2O_2 itself not very reactive, it generates highly reactive molecule such as OH? by reacting with metals (Fe^{2+} or Cu^{2+}), and superoxide anions in the Haber-Weiss reaction. Therefore, removing of H_2O_2 is very essential from the cell or food systems. A significant dose dependent H_2O_2 scavenging potential of catechu bark extract was observed during the present study ($P < 0.05$). Electronic donors might accelerate the conversion of H_2O_2 to H_2O,[13] which could possible to scavenge H_2O_2 in the aqueous extract of bark of *Acacia catechu*. The IC_{50} value of the bark extract was found to be 455.2 ± 15.48 µg/ml whereas that of standard ascorbic acid was 185.24 ± 12.38 µg/ml.

Fig. 3: H_2O_2 scavenging activity of *Acacia catechu* bark extract and the reference compound ascorbic acid.



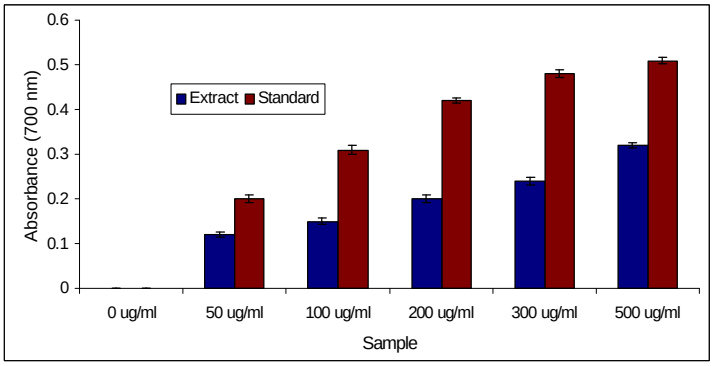
Each value represents mean ± S.E.M. (n=6)

Reducing Power

The reducing ability of a compound generally depends on the presence of reductants,[22] which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom.[23] The presence of reductants (i.e. antioxidants) in *Acacia catechu* extract causes the reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Fig. 4 shows the reductive

capabilities of the plant extract compared to ascorbic acid. The reducing power of extract of *Acacia catechu* was very potent and the power of the extract was increased with quantity of sample. The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the reference standard of ascorbic acid.

Fig. 4: The reductive ability of *Acacia catechu* bark extract and the reference compound ascorbic acid.



Each value represents mean ± S.E.M. (n=6)

Total phenolic content

Phenolic compounds commonly found in all sort of plants and are responsible for multiple biological effects, including antioxidant properties. The total phenolic contents in *Acacia catechu* bark extract was estimated 67.40 ± 0.279 mg/ml gallic acid equivalent per 100 mg plant extract.

Phytochemical Screening

The results of the phytochemical screening of the aqueous extract of *Acacia catechu* are summarized. The screening tests of the aqueous extract were positive for alkaloids, tannins, coumarins, carbohydrates and flavonoids and negative for steroids, anthrones, terpenoids and amino acids.

***In vivo* Antioxidant assay**

Effect on enzyme activity

The oral administration of plant extract to normal rats for one month significantly enhanced the activity of SOD in a dose dependent manner. The results are significant (P<0.001 at the dose of 200 mg/kg body weight and P<0.05 at the dose of 150 mg/kg body weight) when compared with control (Table 1). The treatment induced catalase activity significantly (P<0.001) at the dose of 200 mg/kg body weight (Table 1). The level of GST was also increased significantly (Table 1) at the dose of 150 and 200 mg/kg body weight (P<0.01 and P<0.001, respectively compared with control). The dose-dependent augment of GSH content (Table 1.) was also found to be significantly increased (P<0.001 at 150 mg/kg and 200 mg/kg, p<0.01 at 100 mg/kg body weight).

Toxicity analysis

Table 2 shows the data from the clinical blood chemistry. The increase of serum transaminase enzymes (ALT and AST) levels is a good indicator of hepatocyte damage. No significant changes found in AST levels in the serum of rats. The ALT levels in extract treated rats were found to be increased as compared to control. Nonetheless, these values lie within the normal range. In other parameters like creatinine and Blood Urea Nitrogen (BUN) also, there were no significant changes observed.

Table 2. Effect of the aqueous extract of *Acacia catechu* bark on serum biochemical parameters in Wistar rats

		Aqueous extract			
	Control	50mg/kg	100mg/kg	150mg/kg	200mg/kg
ALT (U/L)	38.2 ± 3.3	36.7 ± 4.5	32.9 ± 5.32	34.8 ± 4.39	41.6 ± 3.25
AST (U/L)	36.1 ± 4.3	38.5 ± 4.25	34.6 ± 6.43	33.8 ± 4.26	32.5 ± 2.87
ALP (U/L)	47.7 ± 4.7	45.2 ± 5.2	43.5 ± 6.3	44.8 ± 4.87	44.9 ± 4.59
BUN (mg/dL)	5.32 ± 0.57	5.87 ± 0.46	5.42 ± 0.63	4.98 ± 0.38	5.12 ± 0.69
Creatinine (mg/dL)	1.78 ± 0.04	1.56 ± 0.24	1.98 ± 0.15	1.68 ± 0.25	1.50 ± 0.32

The values are mean ± SEM. (n=6).
ALT - Alanin aminotransferase
AST - Aspartate aminotransferase
ALP - Alkaline phosphatase
BUN Blood urea nitrogen

Table 1. Effect of the aqueous extract of Acacia catechu bark on the activities of antioxidant enzymes and reduced glutathione content in liver of normal rats

	Control	Aqueous extract 50mg/kg	100mg/kg	150mg/kg	200mg/kg
Reduced Glutathione[a]	2.57±0.04	5.47±0.06*	7.65±0.08**	8.78±0.07***	9.67±0.09***
Glutathione S transferase[b]	2.34±0.05	2.67±0.04	3.32±0.06	3.89±0.06*	4.23±0.08***
Superoxide dismutase[c]	0.63±0.001	0.65±0.002	0.70±0.001	0.76±0.001	0.82±0.001*
Catalase[d]	5.89±0.07	6.31±0.1	7.51±0.09*	8.92±0.1**	9.56±0.06***

[a]nmol of GSH/mg protein
[b]nmol of CDNB conjugate/min/mg protein
[c]nmol of NADPH oxidized/min/mg protein
[d]umol of H_2O_2 consumed/min/gwet wt tissue
Values are expressed as mean ± SEM (n=6), *P <0.05, **P <0.01, ***P <0.001 compared with control

DISCUSSION

Due to increased exposure to environmental damage, our endogenous antioxidant defense system is not completely effective. It seems reasonable to propose that antioxidants are very important in diminishing the cumulative effects of oxidative damage. Although, all hu-

man cells protect themselves against oxidative damage by some antioxidant mechanism, these sometimes are not sufficient to prevent the ROS damage totally. Different kind of plant materials have already been reported as natural antioxidants. The present study is focussed on the evaluation of antioxidant activity of the aqueous extract of bark of *Acacia catechu*, *in vitro* as well as *in vivo*.

Antioxidants, when present in trace levels, the response of antioxidants to different radical or oxidant sources may be different. Therefore, no single assay accurately reflects the mechanism of action of all radical sources or all antioxidants in a complex system,[24] at least two methods should be employed in order to evaluate the total antioxidant activity,[25] due to various oxidative processes. The antioxidant activity of a compound has been attributed to various mechanisms viz. prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging ability.

The total antioxidant activity by Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid. The high absorbance values indicated that the aqueous extract of bark of A. catechu possessed significant antioxidant activity (Fig. 1.). DPPH is a stable radical that has been used to evaluate the antioxidant activity of plant and microbial extracts. In the current study, DPPH scavenging activity of the bark extract was found to be comparable with ascorbic acid. Hydrogen peroxide, a weak oxidizing agent can cross cell membrane rapidly, reacts with Fe^{2+} and possibly Cu^{2+} ions to form the damaging toxic hydroxyl radical. The scavenging activity of the aqueous bark extract was found to be dose dependant and the IC_{50} value was found to be 455.2 ± 15.48 µg/ml.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. As shown in Fig. 4, it may be observed that the plant extract has some reducing capacity, thus justifying its antioxidant capacity. It is also found that *Acacia catechu* bark extract shows significant amount of phenolic content. Phenolic compounds are known powerful chain breaking antioxidants. They are considered as important plant constituents because of their scavenging ability due to their hydroxyl groups and contribute directly to antioxidative action.[26] Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants.[27] It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily with a diet rich in fruits and vegetables.[28] In the present study, the total phenolic content was 67.40 ± 0.279 mg/ml gallic acid equivalent per 100 mg plant extract for the aqueous extract of bark of *Acacia catechu*. The antioxidant activity of phenolic compounds is mainly attributed for their redox actions, neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. In the phytochemical analysis the aqueous extract showed presence of alkaloids, tannins, coumarins, carbohydrates and flavonoids.

According to the above results, the aqueous bark extract of *A. catechu*, showed excellent antioxidant activity *in vitro*. To further evaluate the antioxidative properties of bark extract on living cells, *in vivo* antioxidant activity measurement was employed and found that the extract is equally effective in combating oxidative stress in the living system without producing any signs of toxicity.

It has been reported that body's antioxidant defense system consisting of the activity of SOD, CAT, GST and GSH.[29] SOD catalyzes the breakdown of endogenous cytotoxic superoxide radicals to H_2O_2 which is further degraded by CAT. Thus, they play a crucial role in maintaining the physiological levels of O_2 and H_2O_2. GSH, in conjunction with GST, has a basic role in cellular defense against deleterious free radicals and other oxidant species.[30] GST catalyzes the conjugation of thiol group of glutathione to electrophilic substrates, and thereby detoxifies endogenous compounds such as peroxidized lipids.[31] The present study supports the antioxidant potency of the plant extract as evidenced by the increased level of these antioxidant systems in extract treated rats.

The extract was further tested for its toxicity using clinical blood chemistry. The increase of serum transaminase enzymes (ALT and AST) levels is a good indicator of hepatocyte damage.[32] ALT and AST are two liver enzymes that are associated to the hepatocellular damage. Although both AST and ALT are common liver enzymes because of their higher concentrations in hepatocytes, only ALT is remarkably specific for liver function and considered a highly sensitive indicator of hepatotoxicity,[33] since AST is mostly present in the myocardium, skeletal muscle, brain and kidneys. There were no significant changes found in these enzyme levels when compared with the control. The blood urea nitrogen test is a measure of the amount of nitrogen in the blood in the form of urea, and a measurement of renal function. Abnormally high levels of creatinine thus warn of possible malfunction or failure of the kidneys. All the levels were found with in normal range.[34] These results indicated that this extract when taken for long periods of time might not cause liver or renal disease.

CONCLUSION

In conclusion, the present study provides evidence that aqueous extract of bark of *Acacia catechu* shows potential antioxidant and free radical scavenging activity. These *in vitro* assays demonstrate that plant extracts are important sources of natural antioxidants, which might be useful as preventive agents against oxidative stress. Furthermore, evaluation of *in vivo* antioxidant activity and toxicity of this extract has also provided interesting results that might be beneficial for the pharmacological use of this plant in clinical trials. In a word, these results signify that *Acacia catechu* bark extract is an important source of natural antioxidant, which might play a vital role in preventing the progress of various oxidative stresses, in course of enhancing the generation of typical antioxidant enzymes. To elucidate the prime source of antioxidant properties further studies need to be carried out with the active principles and subsequently reported at a later date.

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