

**ANTIMICROBIAL, PHYTOCHEMICAL AND ANTIOXIDANT SCREENING
OF LEAVES AND STEM BARK FROM *ALBIZIA LEBBECK* (L.)****SANDHYA MALLA, C. K. SHROTRI AND REENA JAIN****Department of Lifescience & Technology, Boston College for Professional Studies, Gwalior***ABSTRACT**

Shirish (*Albizia lebbbeck*) leaves and bark have been used in traditional medicines for treatment of inflammatory diseases. The objective of this work was to evaluate the antimicrobial activity, phytochemical screening and antioxidant property of methanol soluble extracts of leaves (MSL) and bark (MSB). Best antimicrobial activity was observed with methanol soluble bark against both *E. coli* and *S. aureus*. The analysis of minerals in leaves revealed high amount of mineral elements. The potassium content was highest (578.30 mg/100g dry wt.) followed by sodium, calcium, magnesium, iron, zinc and copper. Total phenolics and anthocyanins were higher in MSL. The flavanoid to phenolics ratio (F/P ratio) were 0.428 and 0.072 in MSL and MSB respectively. Antioxidant studies revealed the IC₅₀ values 240, 100, 160 and 135µg/ml for DPPH scavenging, deoxyribose degradation, nitric oxide and H₂O₂ scavenging assays, respectively which were comparable to the IC₅₀ of values standard ascorbic acid. The plant *Albizia lebbbeck* was found to be a rich source of phyto-constituents having immense antioxidant potential and the leaves as good source for mineral nutrients.

KEY WORDS: antimicrobial, phytochemical, shirish, *Albizia lebbbeck***REENA JAIN**

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INTRODUCTION

Albizia lebbek (Shirish, common name) is a tree well known in the Indian subcontinent for its medicinal uses. It is used in the Indian traditional system and folk medicine as well to treat several inflammatory pathologies such as asthma, arthritis and burns¹. The alcoholic extract of this plant has antihistaminic property, by neutralizing the histamine directly or due to corticotrophin action as evidenced by raising cortisol levels in plasma². Saponins of *A. lebbek* has been claimed to be useful in the treatment of Alzheimer's and Parkinson's diseases³. The majority of the diseases or disorders are mainly linked to oxidative stress due to free radicals⁴. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism⁵. The most common reactive oxygen species (ROS) include super oxide anion, hydroxyl, hydrogen peroxide and peroxy radicals. The nitrogen derived free radicals are nitric oxide and proxy nitrite anion⁶. ROS have been implicated in over a hundred of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and cardio vascular malfunction⁷. In treatments of these diseases, antioxidant therapy has gained an immense importance. The present study was planned for the assessment of *Albizia lebbek* antioxidant property, antimicrobial activity with preliminary phytochemical characterization.

MATERIALS AND METHODS

(i) *Determination of mineral elements in plant leaves*

The mineral elements of plant leaves were determined in the acid digested powdered leaf samples by Atomic Absorption Spectrophotometer (AS-72, AAS PerkinElmer, Norwalk, CT, USA)⁸. Plant leaves were washed with deionised water, dried at 60°C and ground in to fine powder. One gram powdered sample was allowed for wet-digestion. After acid-digestion, allowed for cooling the samples. The cooled sample was filtered. The filtrate was

used for the final mineral analysis by Atomic absorption spectroscopy. The results were expressed as mg per gram dry weight.

(ii) *Preparation of the plant extract*

The plant leaves and stem bark of *Albizia lebbek* were collected from Jiwaji University, Gwalior campus and surface sterilized using 1% HgCl₂ and phosphate buffer saline, shade dried at room temperature and powdered mechanically. Fifty grams of dried bark and leaves were soaked in individual solvents, autoclaved hot triple distilled water and methanol, under constant stirring. The filtrate was collected three times at 24 h intervals during a total extraction period of 72 h. The aqueous and methanol dry extracts were obtained by concentrating the extract liquid under reduced pressure at 40°C using a vacuum rotary evaporator. The dry extracts were stored at -20 °C until use.

(iii) *Anti-Microbial Activity*

To check antimicrobial activity minimum inhibitory concentrations of (MIC) of different extracts were determined against *Escherichia coli* and *Staphylococcus aureus* using the test tube dilution method^{9, 10}. Each of the extracts was constituted by dissolving 1.0 g of the concentrates in 10 ml of nutrient broth, making the concentration to be 100 mg/ml. Fifteen tubes of 5 ml of nutrient broth were set up, and appropriate amount of the 100 mg/ml of the extracts were added to different fifteen tubes of the nutrient broth to give the concentrations of extracts ranging from 100 mg/ml to 2.5 mg/ml. Normal saline was used to prepare a turbid suspension of test bacteria. The dilution of the test bacteria was done continuously in the normal saline until the turbidity matched that of 0.5 Mc-Farlands standard by visual comparison. At that point, microorganism has a concentration of about 1.5×10^8 cfu/ml. 0.1 ml of this suspension was transferred into the test tubes containing broth with different concentrations of extracts¹⁰. The tubes were incubated at 37°C for 24 h. The minimum inhibitory concentration was regarded as the

lowest concentration that inhibited the visible growth.

(iv) Phytochemical screening

Preliminary phytochemical screening of the powdered leaves and bark were performed for the presence of alkaloids, carbohydrates, reducing sugars, proteins, flavonoids, tannins, phenols, phytosterol and saponins using the standard procedures described.^{11, 12.}

Alkaloids

Alkaloids were detected by using Wagner's test. To 1 ml of extract, 2-3 drops of wagner's reagent was added. Appearance of reddish brown precipitate indicated the presence of alkaloids.

Saponins by Froth test

Extracts were diluted to 20 ml with distilled water and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

Steroids by Salkowski test

To the few mg of extract, 2 ml of chloroform and 2 ml of conc. sulphuric acid was added. Tubes were shaken and allowed to stand. A golden yellow red colour indicated the presence of phytosterols.

Tannins

Few drops of 1% lead acetate were added to 2 ml of extract. The formation of yellowish precipitate indicated the presence of tannins.

Tannins (Ferric Chloride Test)

Extract solutions were treated with 5% ferric chloride solution. As per Culet *et al.*, (2010) formation of blue colour indicated the presence of hydrolysable tannins and formation of green colour indicated the presence of condensed tannins¹³

Flavanoids

As described by Edeoga *et al.*, (2005), a portion of the powdered plant sample was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed, indicating a positive test for flavanoids.¹⁴

Carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were

used for Molisch's test for the presence of carbohydrates and Fehling's test for the presence of reducing sugars.

Proteins (Biuret's Test and Ninhydrin Test)

To 1 ml of test extract, 4% of sodium hydroxide solution and few drops of 1% copper sulphate solution were added. Formation of a violet red colour indicated the presence of proteins.

To 2 ml of test extract, few drops of 0.25% of ninhydrin in acetone were added and heated in boiling water bath for 10 min. The formation of bluish purple colour indicated the presence of amino acid or proteins.

Estimation of total phenolic content

The total phenolic content in extracts was determined with slight modifications of Folin-Ciocalteu method¹⁵. An aliquot of the extract in ethanol: triple distilled water (1:1) was treated with 5 ml Folin-Ciocalteu reagent (diluted with 1:10 v/v) and 4 ml of 1 M sodium carbonate. The tubes were incubated at room temperature for 15 min and centrifuged. The absorbance of supernatant was measured against blank at 765 nm. An equivalent amount of distilled water instead of extract was added in blank. Results were expressed as mg/g Gallic acid equivalent.

Estimation of total Flavonoids

The total Flavonoids content was estimated by aluminum chloride colorimetric method given by Woisky and Salatino¹⁶ with slight modifications. The standard calibration curve was prepared from (25µg/ml-200µg/ml) Quercetin. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Then the reaction mixture is incubated at room temperature for 30 min. After that the absorbance was measured at 415 nm. In blank, 0.1 ml of distilled water was added instead of 10% aluminum chloride. The amount of flavanoids was expressed as mg quercetin equivalent per gm dry weight of extract.

Estimation of Anthocyanin content

The total monomeric anthocyanin in different samples was determined using the principle of reversible structural transformations with a change in pH¹⁷. The colored oxonium form

predominates at pH 1.0 and the colorless hemiketal form at pH 4.5. The dilution factor for the sample was determined by diluting the sample with 0.025M potassium chloride and measuring absorbance at 530nm. The absorbance at 530 nm should be less than 1.2. Two dilutions were performed on each sample. The first used potassium chloride (0.025 M) at pH 1 and the second was with sodium acetate (0.4 M) at pH 4.5, and allowed to equilibrate for 15 minutes¹⁸. Absorbance was measured at 530 nm and 700 nm against blank. The difference in absorbance between pH values and wavelengths were calculated by

$$A = (A_{530\text{nm}} - A_{700\text{nm}}) \text{ pH}1.0 - (A_{530\text{nm}} - A_{700\text{nm}}) \text{ pH}4.5$$

The concentration of monomeric anthocyanin pigment was obtained by

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times 1)$$

Where MW is the molecular weight, DF is the dilution factor, ϵ is the molar absorptivity, and 1 is for a standard 1-cm path length. The molecular weight (MW = 449.2) and molar absorptivity ($\epsilon = 26900$) for cyanidin-3-glucoside was used. The total monomeric anthocyanins were reported as milligrams anthocyanins per 100 g dry weight (mg cyanidin-3-glucoside/100 g fw or dw).

(v) Determination of antioxidant activity (In vitro antioxidant activity)

DPPH radical scavenging assay

The hydrogen donating ability of the extracts was determined in the presence of DPPH (1,1 diphenyl-2-picrylhydrazyl) stable radical¹⁹. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentration (100 μ g-500 μ g) and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm. Ethanol (1.0 ml) plus plant extract solution (2.5ml) was used as a blank, DPPH solution (1.0ml, 0.3mM) plus ethanol (2.5ml) served as negative control. The positive controls were those using the standard ascorbic acid solutions.

Deoxyribose degradation assay (Hydroxyl Radical Scavenging Activity)

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al.*²⁰. Stock solutions of EDTA (1mM), FeCl₃ (10 mM), ascorbic acid (1mM), H₂O₂ (10mM) and deoxyribose (10mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml of EDTA, 0.01 ml of FeCl₃, 0.1 ml of H₂O₂, 0.36 ml of deoxyribose, 1.0 ml of plant extract (100-500 μ g/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1hr. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA to develop the pink chromogen, measured at 532 nm. The hydroxyl radicals scavenging activity was calculated using the following equation²¹

$$\% \text{ Inhibition} = [(A_c - A_s) / A_c \times 100]$$

Where, A_c was the absorbance of the control (blank) and A_s was the absorbance in the presence different concentrations of the extract.

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity was determined by the method of Sreejayan²², (1997). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions²³. The produced NO can be estimated by Griess's reagent. The production of nitrite ions are reduced in presence of nitric oxide scavengers. 2 ml of 10 mM sodium nitroprusside in 0.025 M phosphate buffer (7.4), 0.5 ml of 0.025M phosphate buffer (7.4) and 0.5 ml of various concentration of extract were mixed and incubated at 25°C for 150 min. In 0.5 ml of incubated mixture, 0.5 ml of Griess's reagent was added (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in triple distilled water). The absorbance of the chromophore formed during the diazotisation of nitrite with Griess's reagent was measured at 546 nm. The control contains the equivalent amount of buffer in place of test sample. Ascorbic acid was taken as standard. The percentage inhibition was calculated.

Hydrogen peroxide scavenging activity

The percentage scavenging activity of hydrogen peroxide was determined by the method described by Kaser, *et al.*²⁴. A solution of 40mM hydrogen peroxide was prepared in 50 mM phosphate buffer (pH 7.4). One ml of various concentration of extract and 0.6 ml of 40 mM hydrogen peroxide were mixed and incubated at room temperature for 10 minutes. After incubation, absorbance was measured at 230 nm against blank containing phosphate buffer without hydrogen peroxide. Ascorbic acid was taken as standard. Percentage scavenging activity was calculated.

Reducing power ability

Reducing power ability of plant extract was determined by the method described by Oyaizu²⁵. Different concentrations of the extract were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and cooled. 2.5 ml of 10% of trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of freshly prepared 0.1% FeCl₃ and allowed to stand for 10 min. The absorbance was measured at 700 nm. The increased absorbance indicated high reducing power. Ascorbic acid was used as reference standard.

Calculation of 50% inhibitory concentration (IC50)

The Concentration ($\mu\text{g/ml}$) of the extract required to scavenge 50% of the radicals was calculated by using the graph between percentage inhibition (scavenging activities) at five different concentrations of the extract.

RESULTS AND DISCUSSION**1. Mineral composition of Leaves**

Data on mineral elements of *A. lebbeck* leaves are presented in Table 1. Leaves of *Albizia lebbeck* recorded the high mineral content. The potassium content is highest followed by sodium, calcium, magnesium, iron, zinc and copper. Essential trace minerals such as Zinc (Zn), Copper (Cu) and Iron (Fe) are known to play important role in the maintenance of redox homeostasis. Alterations in the status of these minerals may result in stronger inflammatory responses and increased oxidative stress. Zn is critical to the structure of SOD and can stabilize biological membranes to decrease their susceptibility to oxidative damage. Cu is required for the catalytic activity of SOD, and Cu deficiency or excess can induce oxidative stress that can lead to chronic inflammation and affect immune responses. Fe is a constituent of enzyme catalase. Free Fe can participate in the Fenton reaction, catalyzing the generation of hydroxyl radicals²⁶. The *A. lebbeck* leaves being good natural sources of minerals can be used to trace element deficiency.

Table 1
Minerals of *A. lebbeck* leaves

Mineral element	Amount in mg / g dry wt.
Calcium Ca	157.80
Iron Fe	39.12
Potassium K	578.30
Magnesium Mg	141.50
Copper Cu	0.08
Zinc Zn	5.4
Sodium Na	263.42

2. Anti-Microbial Activity

The minimum inhibitory concentration (MIC) of both the methanol and aqueous extracts of bark and leaves are given in Table 2. Best antimicrobial activity was observed with Methanol soluble bark against both *E. coli* and *S. aureus*. On preliminary screening methanol soluble bark seems to effective against both Gram positive and Gram negative bacteria. However, needs to be tested for antibacterial activity with other bacterial species.

Table 2
Anti-Microbial activity of different Extracts

S.No	Extracts	MIC (mg/ml)	
		<i>E. coli</i>	<i>S. aureus</i>
1.	MSB	1.0	2.5
2.	ASB	2.5	2.5
3.	MSL	100.0	100.0
4.	ASL	50.0	75.0

3. Phytochemical screening

Phytochemical screening of the plant extracts revealed the presence of alkaloids, saponins, carbohydrates, proteins and tannins (Table 3). Steroids are absent in all extracts except in methanol soluble bark. The reducing sugar was found in all the extracts except in aqueous extract of leaves.

Table 3
Preliminary phytochemical screening of extracts of Bark and leaves

Constituents	Methanol extract	Bark	Aqueous extract	Bark	Methanol extract	leaves	Aqueous extract	Leaves
Alkaloids (Wagner's test)	+		-		+		+	
Carbohydrates (Molisch's test)	+		+		+		+	
Reducing sugar (Fehling's test)	+		+		+		-	
Proteins (Biuret test)	+		+		+		+	
Saponin (Foam test)	++		+		+		++	
Steroids (Salkowski' test)	+		-		-		-	
Tannins	+		+		+		+	
Flavanoids	+		+		+		+	

Data on the total phenolics, flavanoids and anthocyanins contents are presented in Table 4. Total phenolics, flavanoids and anthocyanins are found to be more in bark than in leaves. Moreover, the flavanoids to phenolics ratio is more in methanol soluble extract of leaves.

Phenolic compounds are powerful chain breaking antioxidants²⁷. Herbal derived antioxidants especially polyphenols and flavonoids have been recognised to have anticancer, anti diabetic, anti aging properties and prevention of cardiovascular diseases²⁸.

Polyphenolic and flavanoid compounds contain conjugated ring structures and hydroxyl groups; they function as antioxidants in cell free systems by scavenging singlet oxygen, superoxide anion, lipid peroxy radicals, hydroxyl ions, nitric oxide ions, and stabilizing free radicals involved in oxidative processes²⁹. The total phenolic contents of MSL were found 52.48 mg/g (5.248%) which was equivalent, to

Gallic acid. Flavanoids are also potential antioxidants³⁰. The total flavanoid contents of MSL was found 2.248% and equivalent to quercetin. Flavanoids have been reported to have hypoglycemic effect³¹. It is suggested that the leaves can be used for the management of diabetes. However, the studies are required in this aspect.

Table 4

Concentration of phenolics, flavanoids and anthocyanins in *A. lebbbeck* bark and leaves

Plant Sample	Phenolics	Flavanoids	F/P ratio	Anthocyanins
MSB	36.66 ±1.10	2.65 ±0.01	0.072	0.2003± 0.03
MSL	52.48 ± 4.63	22.48 ±1.81	0.428	6.96± 1.3

Note:

- Phenolics expressed as mg Gallic acid equivalent/ gm of dry powder
- Flavonoids expressed as mg Quercetin equivalent/ gm of dry powder
- milligrams anthocyanins per 100 g dry weight (mg cyanidin-3-glucoside/100 g dw).
- Values expressed as mean ± S.D.

4. Antioxidant assay

In complex systems, various different mechanisms may contribute to oxidative processes such as generation of different reactive oxygen species from various target structures such as carbohydrates, proteins and lipids. Thus, it is necessary to characterize the plant extracts by different antioxidant assays³².

DPPH radical scavenging assay

The DPPH test provides information on the activity of test compound/extract with a suitable

free radical. The hydrogen donating ability of MSL and MSB extracts were estimated in presence of DPPH stable radical. When DPPH reacts with hydrogen donors, it is reduced to a corresponding hydrazine. The degree of reduction in absorbance measurement by MSL and MSB extracts is indicative of the antioxidant power of the plant. DPPH scavenging data are depicted in Figure 1. The IC₅₀ value for MSL and MSB were found to be 240µg/ml and 260µg/ml which is comparable to the IC₅₀ value for ascorbic acid.

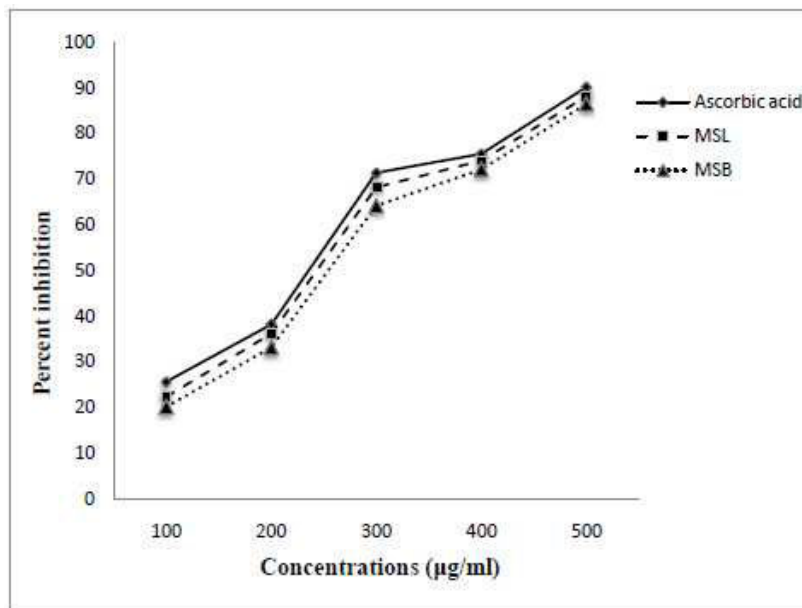


Figure 1
DPPH scavenging activity of *A. lebeck* Extracts

Deoxyribose degradation assay

The hydroxyl radical scavenging activity is measured as the percent inhibition of hydroxyl radicals generated in the Fenton's reaction mixture by studying the competition between deoxyribose and the plant sample extracts for hydroxyl radicals generated from Fe^{+2} /ascorbate /EDTA/ H_2O_2 systems³³. Data on hydroxyl radical scavenging activity are depicted in Figure 2. The IC_{50} value for MSL and MSB were found to be $100\mu g/ml$ and $180\mu g/ml$ which is comparable to the IC_{50} value for ascorbic acid ($60\mu g/ml$).

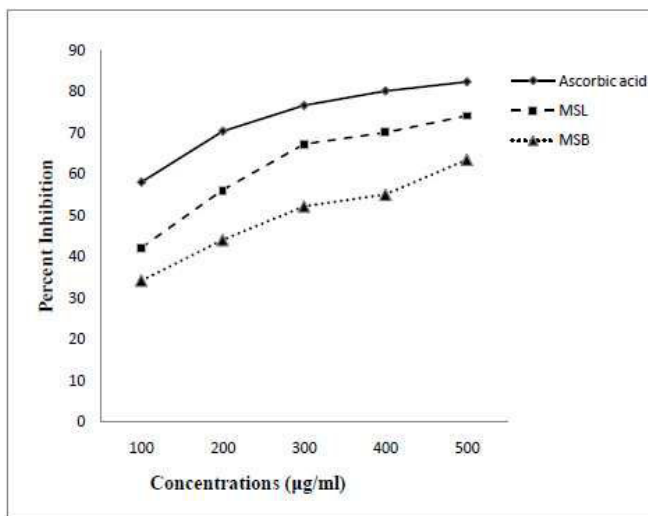


Figure 2
Deoxyribose degradation (Hydroxyl radical) scavenging activity of *A. lebeck* Extracts

Nitric oxide radical scavenging assay

Data on percent inhibition of nitric oxide radical is depicted in Figure 3. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by the contents of MSL and

MSB extract both. The IC₅₀ value for MSL and MSB were found to be 160µg/ml and 270µg/ml respectively. However, the IC₅₀ value with standard ascorbic acid was found to be 90µg/ml.

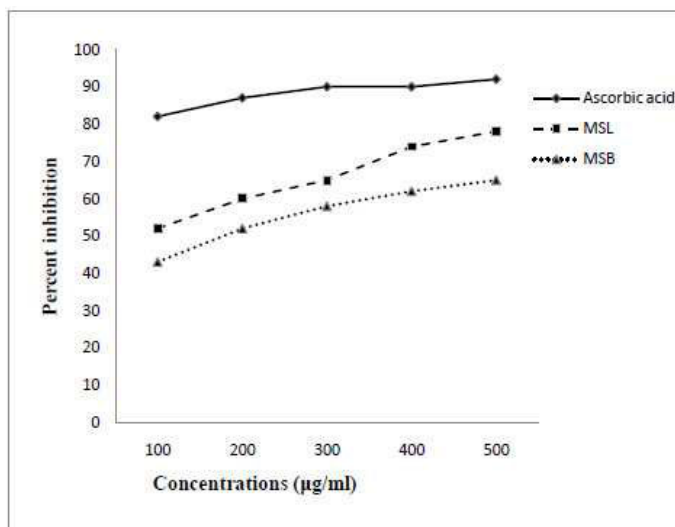
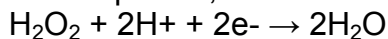


Figure 3

Nitric Oxide radical scavenging activity of *A. lebbeck* Extracts

Hydrogen peroxide scavenging activity

Data on H₂O₂ scavenging activity are presented in Figure 4. The IC₅₀ value for MSL and MSB were found to be 135µg/ml and 255µg/ml respectively. These were comparable to the standard ascorbic acid (95 µg /ml). The decomposition of hydrogen peroxide to water involves the transfer of electrons as in equation,



The scavenging of hydrogen peroxide by phenolic compounds has been attributed to their electron donating ability. The methanol soluble leaf extract has the high electron donating ability.

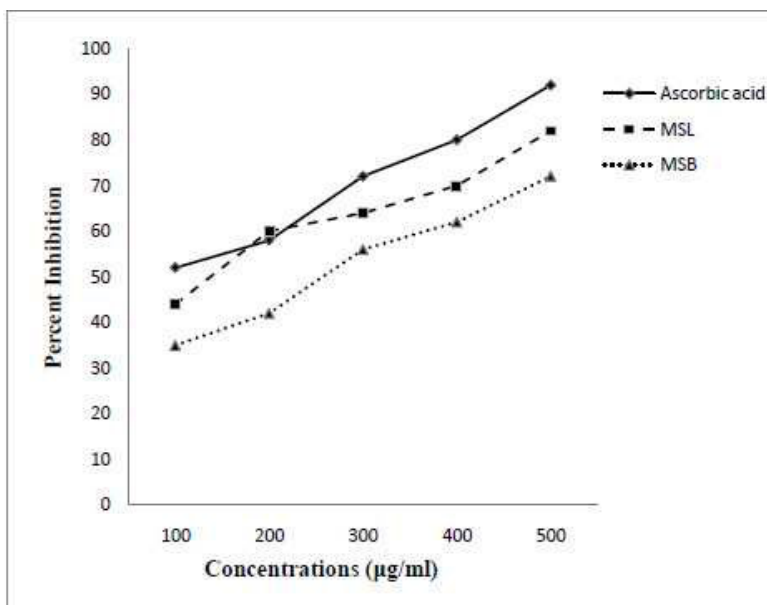


Figure 4

H₂O₂ Scavenging activity of *A. lebbeck* Extracts

Reducing power ability

Data on the total reducible ability of MSL and MSB are presented in Figure 5. Reducing power assay measures the electron-donating capacity of an antioxidant. The reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm, and an increased absorbance is indicative of higher

reducing power. The reducing power of the extract increased progressively over the concentration range studied. MSL extract at 200 $\mu\text{g}/\text{ml}$ had comparable reducing power to Ascorbic acid at 100 $\mu\text{g}/\text{ml}$. These findings suggest that the *A. lebbek* extracts are capable of donating electrons, and could therefore react with free radicals or terminate chain reactions.

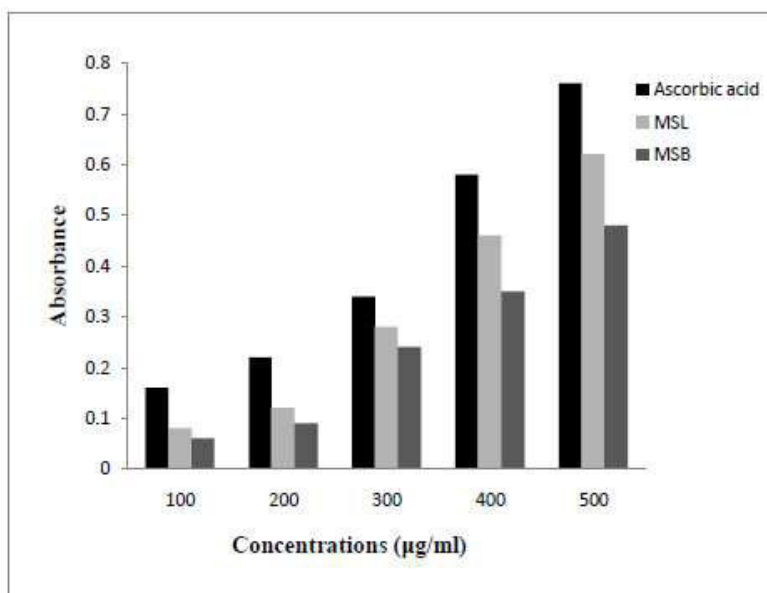


Figure 5
Total reducing ability at 700nm of *A. lebbek* Extracts

CONCLUSION

The present study clearly indicates that *Albizia lebbek* a rich source of phyto-constituents having immense antioxidant potential. The plant is rich in phenolics and flavonoids. The leaves are a good source for mineral nutrients. Since extracts possess good free radical scavenging activity, it would be promising for the development as ingredients of functional foods and pharmaceuticals, for example with diseases preventing such as atherosclerosis, inflammation etc.

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COMPETING INTERESTS

The authors have no financial or non-financial competing interests.

AUTHOR CONTRIBUTIONS

All the authors contributed equally in conceiving, designing and performing the experiments.

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