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# ANTIMICROBIAL, PHYTOCHEMICAL AND ANTIOXIDANT SCREENING OF LEAVES AND STEM BARK FROM *ALBIZIA LEBBECK* (L.)

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## ABSTRACT

Shirish (*Albizia lebbeck*) 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 $IC_{50}$ values 240, 100, 160 and 135µg/ml for DPPH scavenging, deoxyribose degradation, nitric oxide and $H_2O_2$ scavenging assays, respectively which were comparable to the $IC_{50}$ of values standard ascorbic acid. The plant *Albizia lebbeck* 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 lebbeck***

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# INTRODUCTION

*Albizia lebbeck* (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 burns[1]. 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 plasma[2]. Saponins of *A. lebbeck* has been claimed to be useful in the treatment of Alzheimer's and Parkinson's diseases[3]. The majority of the diseases or disorders are mainly linked to oxidative stress due to free radicals[4]. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism[5]. 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 anion[6]. 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 malfunction[7]. In treatments of these diseases, antioxidant therapy has gained an immense importance. The present study was planned for the assessment of *Albizia lebbeck* 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 $60^0$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 lebbeck* were collected from Jiwaji University, Gwalior campus and surface sterilized using 1% $HgCl_2$ 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 x $10^8$ cfu/ml. 0.1 ml of this suspension was transferred into the test tubes containing broth with different concentrations of extracts[10]. The tubes were incubated at $37^o$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 tannins[13]

***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-Ciocalteau method[15]. 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 Salatino[16] 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 pH[17]. 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 minutes[18]. Absorbance was measured at 530 nm and 700 nm against blank. The difference in absorbance between pH values and wavelengths were calculated by

$$A = (A530nm - A700nm)\ pH1.0 - (A530nm - A700nm)\ pH4.5$$

The concentration of monomeric anthocyanin pigment was obtained by

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

Where MW is the molecular weight, DF is the dilution factor, $\varepsilon$ is the molar absorptivity, and 1 is for a standard1-cm path length. The molecular weight (MW = 449.2) and molar absorptivity ($\varepsilon$ = 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 radical[19]. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentration (100µg-500µg) 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), $FeCl_3$ (10 mM), ascorbic acid (1mM), $H_2O_2$ (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 $FeCl_3$, 0.1 ml of $H_20_2$, 0.36 ml of deoxyribose, 1.0 ml of plant extract (100-500 µg/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 equation[21]

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

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 Sreejayan[22], (1997). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions[23]. 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 nitropruside 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$^o$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% napthylethylene diamine dihydrochloride in triple distilled water). The absorbance of the chromophore formed during the diazotisation of nitrite with Greiss'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 Oyaizu[25]. 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$^{o}$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% $FeCl_3$ 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 (µg/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, catalizing the generation of hydroxyl radicals[26]. 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) | |
|---|---|---|---|
| | | *E. coli* | *S. aureus* |
| 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 Bark extract | Aqueous Bark extract | Methanol leaves extract | Aqueous Leaves extract |
|---|---|---|---|---|
| **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 antioxidants[27]. Herbal derived antioxidants especially polyphenols and flavonoids have been recognised to have anticancer, anti diabetic, anti aging properties and prevention of cardiovascular diseases[28].

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 processes[29]. The total phenolic contents of MSL were found 52.48 mg/g (5.248%) which was equivalent, to Gallic acid. Flavanoids are also potential antioxidants[30].The total flavanoid contents of MSL was found 2.248% and equivalent to quercetin. Flavanoids have been reported to have hypoglycemic effect[31]. 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. lebbeck 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 assays[32].

### *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 $IC_{50}$ value for MSL and MSB were found to be 240µg/ml and 260µg/ml which is comparable to the $IC_{50}$ value for ascorbic acid.

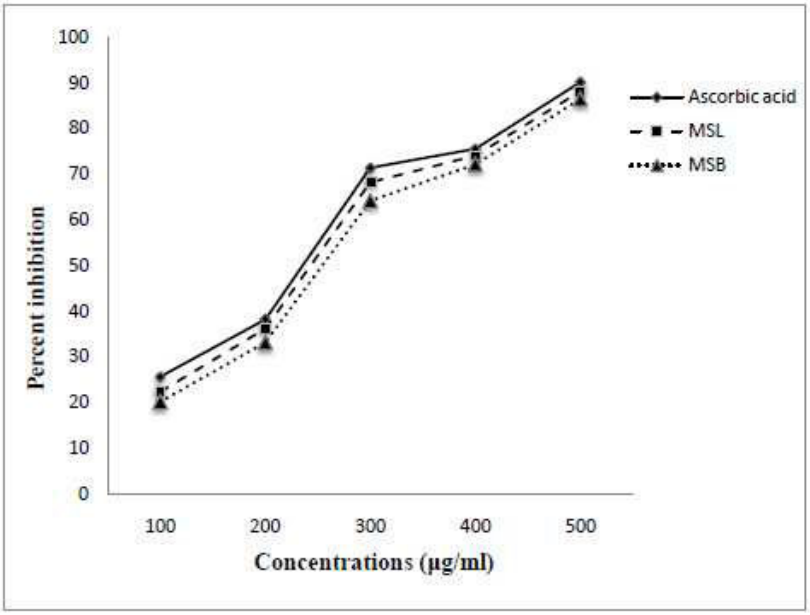


**Figure 1**
***DPPH scavenging activity of A. lebbeck Extracts***

***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/$H_2O_2$ systems[33]. Data on hydroxyl radical scavenging activity are depicted in Figure 2. The $IC_{50}$ value for MSL and MSB were found to be 100µg/ml and 180µg/ml which is comparable to the $IC_{50}$ value for ascorbic acid (60µg/ml).

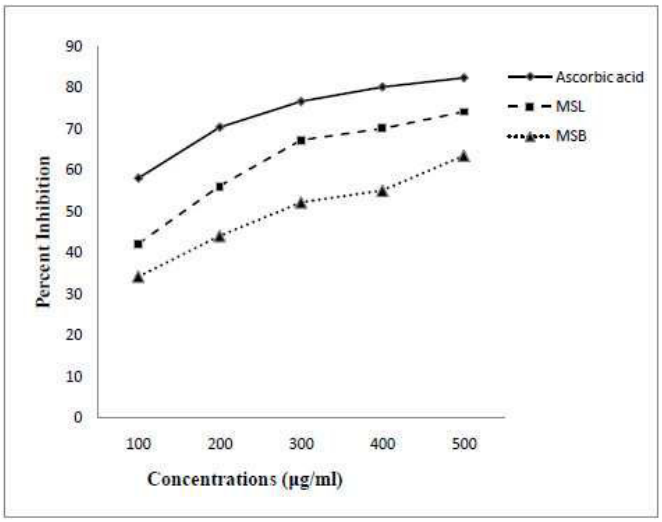


**Figure 2**
***Deoxyribose degradation (Hydroxyl radical) scavenging activity of A. lebbeck Extracts***

***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 $IC_{50}$ value for MSL and MSB were found to be 160µg/ml and 270µg/ml respectively. However, the $IC_{50}$ value with standard ascorbic acid was found to be 90µg/ml.

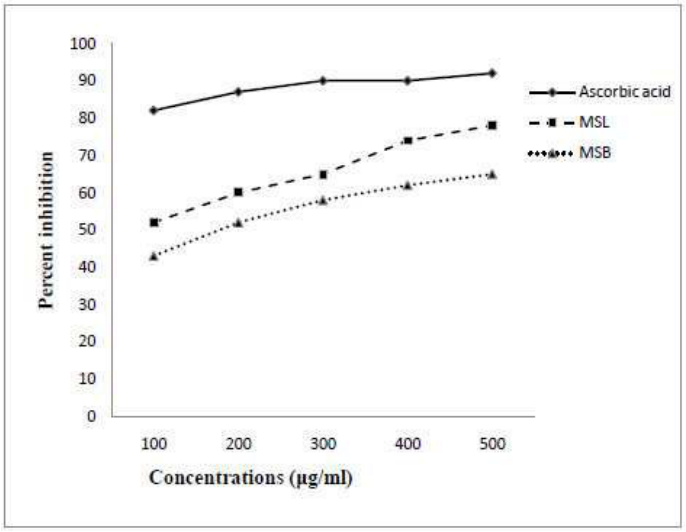


**Figure 3**
***Nitric Oxide radical scavenging activity of A. lebbeck Extracts***

***Hydrogen peroxide scavenging activity***

Data on $H_2O_2$ scavenging activity are presented in Figure 4. The $IC_{50}$ 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,

$H_2O_2 + 2H+ + 2e- \rightarrow 2H_2O$

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.

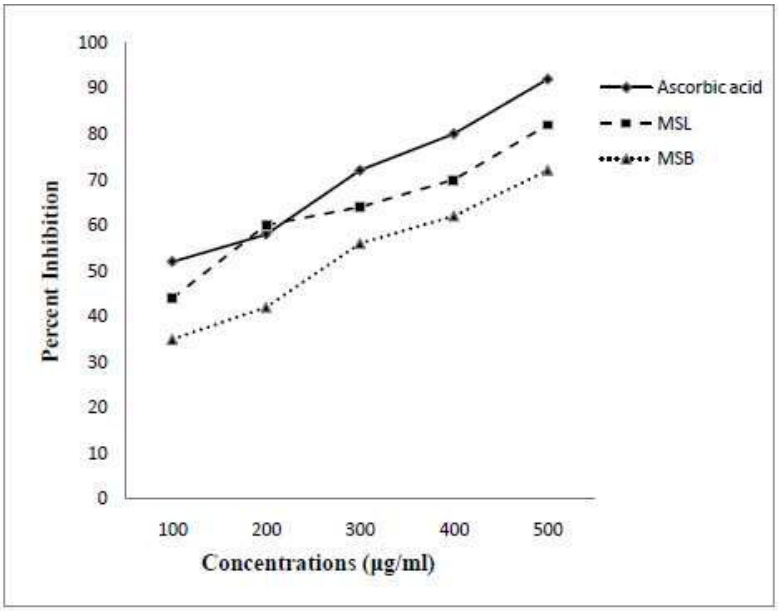


**Figure 4**
***$H_2O_2$ Scavenging activity of A. lebbeck Extracts***

***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 (Fe3+) to ferrous ion (Fe2+) 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 200µg/ ml had comparable reducing power to Ascorbic acid at 100 µg/ ml. These findings suggest that the *A. lebbeck* extracts are capable of donating electrons, and could therefore react with free radicals or terminate chain reactions.

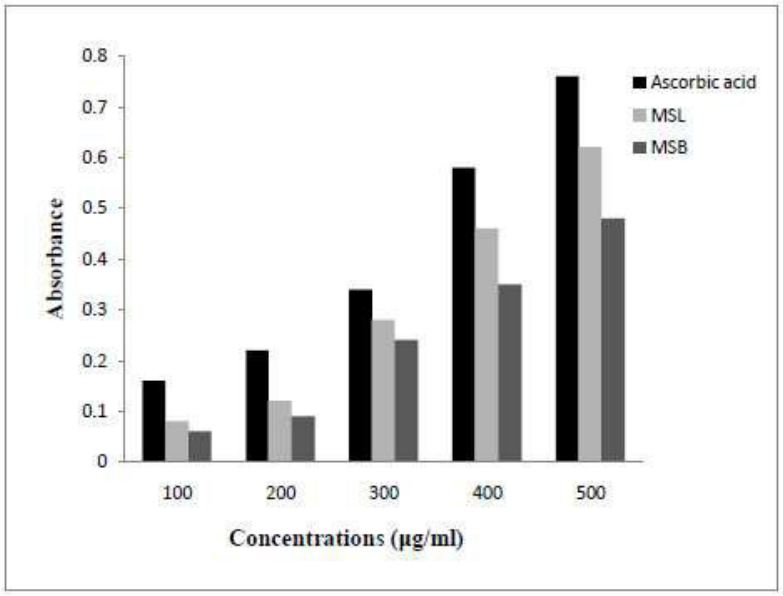


**Figure 5**
***Total reducing ability at 700nm of A. lebbeck Extracts***

## CONCLUSION

The present study clearly indicates that *Albizia lebbeck* 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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