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In-Vitro Evaluation Of Antioxidant Activity Of *Albizia Lebbeck* Bark

P. B. Suruse[1]*, S. B. Bodele[1], N. J. Duragkar[1] And Y. G. Saundankar[1]

*Sharad Pawar College of Pharmacy, Wanadongri, Hingna Road, Nagpur-441 110 (MS), INDIA.

Email : pravin_suruse@rediffmail.com

Corresponding author: Mr. Pravin Babarao Suruse Asstt. Professor Sharad Pawar College of Pharmacy, Wanadongri, Hingna Road, Nagpur-441 110 (MS), INDIA.

Email : pravin_suruse@rediffmail.com

ABSTRACT

The present study was designed to evaluate the *in-vitro* antioxidant potential of methanolic extract and isolated compound from *Albizia lebbeck* bark. The isolation of compound from methanolic extract was carried out by column chromatography technique and purity of isolated compound was checked by thin layer chromatography. The total phenolic contents of methanolic extract and isolated compound were determined by using Folin - Ciocalteu method. The total phenolic content was high in the isolated compound (84.44 mg/g gallic acid equivalent GAE) as compared to methanolic extract (80.25 mg/g gallic acid equivalent GAE). The methanolic extract and isolated compound were investigated for free radical scavenging activity of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) and reducing power assay. The results of DPPH free radical scavenging at 1000 µg/ml indicated maximum antioxidant activity of 91.82% and 90.08% respectively. The reducing power of isolated compound and methanol extract were found to be 2.799 nm and 2.2148 nm respectively. The phytochemical screening revealed the presence of alkaloids and polyphenolic compounds. This suggest a potential utility of the plant as a source of phenolic antioxidants and may provide leads in the ongoing research for natural antioxidants form Indian medicinal plants to be used in treating diseases related to free radical reactions.

Keywords: *Albizia lebbeck,* Free radical scavenging activity, Antioxidant activity.

INTRODUCTION

Antioxidants are believed to quench free radicals free radicals are atom or molecule with singlet unpaired electron which make them highly reactive. Oxidative free radicals are generated by metabolic reactions. Free radicals create a chain reaction leading to membrane lipid peroxidation, DNA damage etc. Free radical oxidation has been implicated in Cancer, atherosclerosis, neurodegenerative diseases and inflammatory bowel disease (Tripathi, 2008). Antioxidants are added to pharmaceutical formulations as redox systems possessing

higher oxidative potential than the drug that they are designed to protect, or as chain inhibitors of radical induced decomposition. In general, the effect of oxidants is to break up the chain formed during a hydrogen atom or an electron to free radical receiving the excess energy possessed by the active molecule (Lachman, 1986).

Albizia lebbeck Linn. **(**Mimosaceae) commonly called as *siris* is widely used in the treatment of many aliments. It has widely distributed all over India, mostly in Maharashtra, Punjab, Gujarat, Karnataka and Madhya pradesh. The bark of *Albizia lebbeck* contains tannins of condensed type, isomers of leucocyanidin, melacacidine, new leucoantho-cyanidin and lebbecacidin. It also contains triedelin and t_3 –sitosterol. The leaves of *Albizia lebbeck* used as an antiseptic and wound healing property. The bark of *Albizia lebbeck* used in the treatment of piles. It also possesses anti-spermatogenic and anti-inflammatory activity (Khare, 2007; Kirtikar, 1999). This study was aimed to investigate antioxidant potential of *Albizia lebbeck bark.* Therefore an effort is made to contribute to establish scientific evidence in this regard.

MATERIALS

Plant material:

The bark of *Albizia lebbeck* was collected from the Botanical garden, Nagpur and taxonomically authenticated from the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. A Voucher specimen (No. 9588) has been deposited in the Herbarium of the same department.

EXPERIMENTAL AND RESULTS

Preparation of extract:

Bark of *Albizia lebbeck* was dried in shade and powdered in hand grinder to get coarse powder. The powdered bark was extracted for 30 h in a Soxhlet apparatus using methanol. The methanolic extract was concentrated at low pressure by distillation and finally air dried.

Preliminary phytochemical screening:

The plants may contain phytochemical constituents like alkaloids, glycosides, sterols, tannins, saponins, sugars, etc. These compounds are secondary metabolites mainly responsible for their physiological and therapeutic effects. The methanol extract and isolated compound from *Albizia lebbeck* bark were subjected to preliminary phytochemical screening for the detection of various plant constituents (Lala, 1985). The results of preliminary phytochemical screening are given in Table 1.

Table 1. Preliminary phytochemical screening of *Albizia lebbeck*

Plant Constituents	Tests	Methanolic extract	Isolated compound
Test for Sterols	Liebermann's test	--	--
	Salkowaski Reaction	--	--
	Liebermann-Burchard Reaction	--	--

Test for Alkaloids	Dragendorff's Reagent	++	++
	Mayer's Reagent	++	++
	Wagner's Reagent	++	++
	Hager's Reagent	--	--
	Tannic acid	++	++
Test for Glycoside	Killer-Killani test	++	++
	Legal test	++	++
	Baljet test	++	++
Test for Flavonoids	Shinoda test	--	--
Test for Saponin	Foam test	++	++
	Heamolysis test	++	++
Test for Tannins	5% $FeCl_3$	++	++
Test for Protiens	Biuret test	++	++
	Million's test	++	++

-- Absent, + Present, ++ Present in higher concentration

Methanolic extract showed positive test for alkaloids which are targeted bioactive constituents hence it was selected for isolation of active constituent. The isolation procedure was carried out by column chromatography method and purity was checked by thin layer chromatography method. The results of thin layer chromatography of methanol extract are shown in Table 2. Mobile phase with Butanol: Glacial Acetic acid: Water (8:1:1) showed maximum resolution and reproductive results. In iodine vapour and after spraying with 50% H_2SO_4 showed 4 spots having R_f values 0.31, 0.49, 0.53 and 0.65.

Table 2: Thin layer chromatography of methanolic extract of *Albizia lebbeck*

Sample spot	**Mobile phase used**	**No. of spots obtained with different locating agent**			**R_f value**
		UV	**Iodine vapour**	**50% H_2SO_4**	
Methanolic extract	Methanol	2	3	2	0.17, 0.23
	Ethyl acetate: chloroform (3:7)	2	3	3	0.34, 0.55, o.67
	Butanol: Glacial acetic acid: Water (8:1:1)	3	4	4	0.31, 0.49, 0.53, 0.65

Column chromatography of methanolic extract:

Preparation of column:

The glass column of about 1.2 m length and 3 cm in diameter was used. The column was cleaned thoroughly using chromic acid and washed with distilled water. Silica gel 60-120 mesh was activated at 120º in hot air oven for 1 h and mixed with petroleum ether to form slurry. The column was fixed vertically on a stand and a cotton plug was inserted to bottom of the column. The column was filled with petroleum ether and the slurry of silica gel was poured slowly from the top. The solvent used for elution was maintained 10 cm above the cotton plug. The methanolic extract (40 g) was triturated with small quantity of silica gel and poured from top of the column. A plug of cotton was placed over it and solvent is maintained 10 cm above the cotton plug (Stahl, 2005; Silverstein, 1991).

Elution of column:

The column was eluted successively with different solvents (from nonpolar to polar) and mixture of solvents, in increasing order of polarity and 30 ml of fractions were collected every time. Eluted fractions were subjected to thin layer chromatography for every solvents or solvent mixture. For homogeneity test of each fraction thin layer chromatogram was run by using different solvent systems and 50% sulphuric acid was used as a detecting reagent. The results of thin layer chromatography of eluted fractions and isolated compound are shown in table 3 and 4 respectively. The result of identification of isolated compound is shown in table 5. The fractions having same TLC pattern were mixed to form single fraction.

Table 3: Thin layer chromatography of eluted fractions

Mobile phase used for TLC	Fraction Number	Iodine vapours	
		No. of Spots	R_f
Petroleum ether	1-12	0	0.00
Benzene	13-18	0	0.00
Benzene: Ethyl acetate (9:1)	19-27	1	0.51
Benzene: Ethyl acetate (8:2)	28-48	1	0.58
Benzene: Ethyl acetate (8:2)	49-68	1	0.62
Benzene: Ethyl acetate(7:3)	69-87	1	0.73
Ethyl acetate	88-101	2	0.76, 0.80
Ethyl acetate: Acetone (9:1)	102-112	2	0.78, 0.80
Ethyl acetate: Acetone (8:2)	113-122	2	0.79, 0.82
Butanol: acetic acid: water (6:1:3)	123-148	1	0.65
Butanol: acetic acid: water (6:1:3)	149-194	1	0.65

Butanol: acetic acid: water (6:1:3)	195-220	1	0.65
Butanol: acetic acid: water (6:1:3)	221-240	1	0.65
Butanol: acetic acid: water (6:1:3)	241-267	1	0.65

Table 4: Thin layer chromatography of isolated compound

Sample spot applied	Mobile phase used	No. of spot obtained with different locating agent		R_f value
		Iodine vapour	Dragendorffs reagent	
Isolated compound	Butanol: acetic acid: water (6:1:3)	1	1	0.65

Table 5: Identification of isolated compound

Parameter	Isolated compound (Alkaloid)
Colour	Cremish brown
Solubility	Methanol
Appearance	Fine powder
Melting Point	144-146°
Chromogenic reagent	Iodine vapour, Dragendorff's reagent
R_f value	0.65

Spectral data of isolated compound:

UV spectra: λ max at 430 nm.

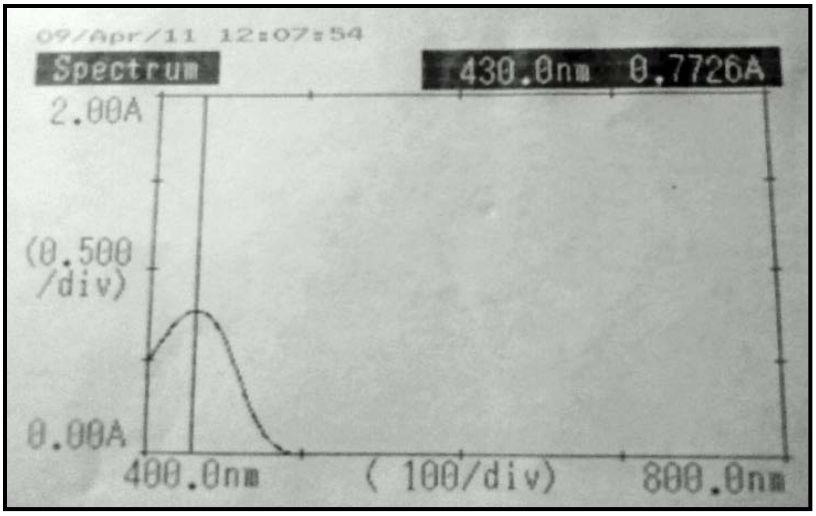


Figure1: UV spectra of isolated compound

IR: ν^{KBr}max (cm^{-1}):3350, 2931, 2360, 2339, 1606, 1639, 1516, 1447, 1360, 1130, 1067, 860, 858, 830, 777, 608.

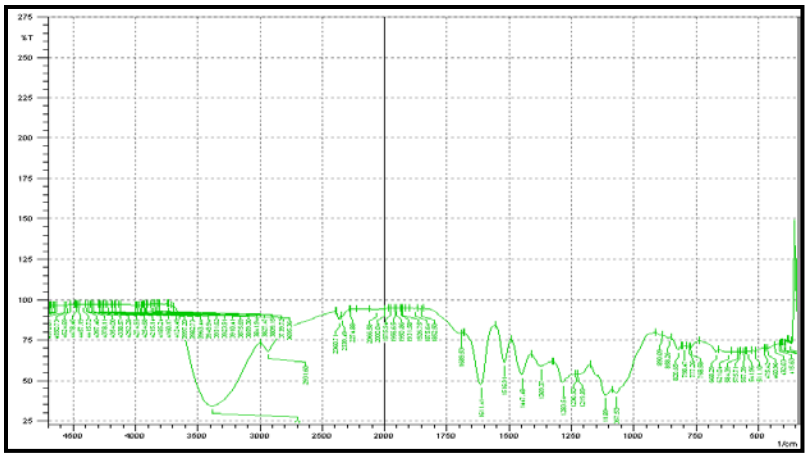


Figure 2: IR spectra of isolated compound

^{1}H NMR:PMR **δ** ppm-7.27(s), 6.82, 6.79(d), 6.73(s), 6.69, 6.62(d), 6.02, 5.98(d), 4.19(s), 3.74, 3.69(d), 3.30(s), 3.05, 3.03, 3.02(t), 2.79, 2.77,2.76(t), 2.48(s).

^{13}C NMR: δ ppm-150, 147, 146, 144, 141, 129, 128, 124, 122, 119, 112, 108, 104, 101, 95, 60, 56, 49, 48, 40, 39, 29.

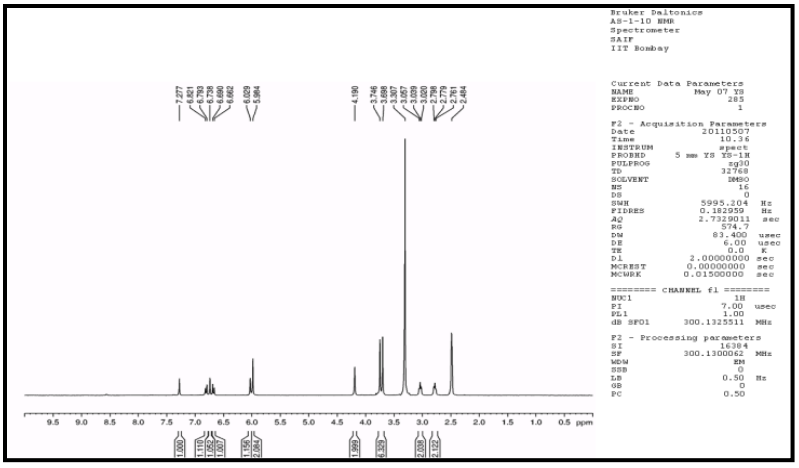


Figure 3: PMR spectra of isolated compound

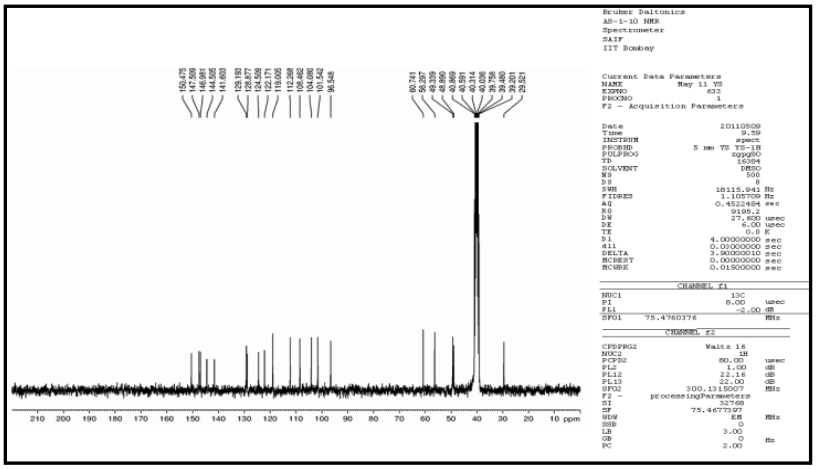
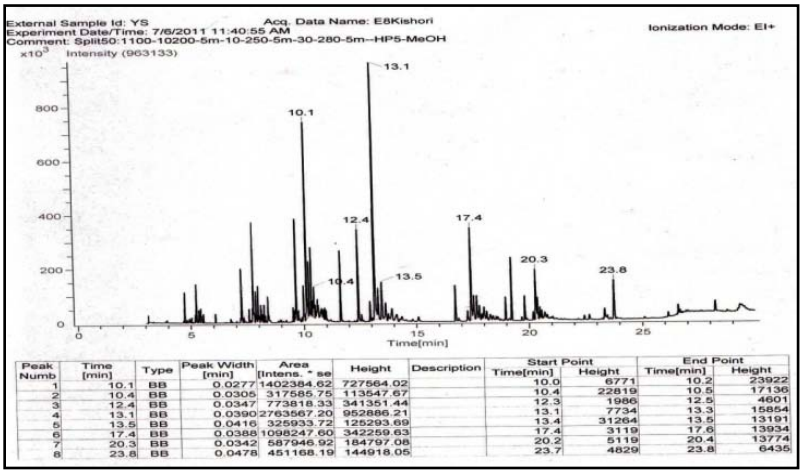


Figure 4: ^{13}C NMR spectra of isolated compound

Mass spectra: Molecular ion peak were observed at M+1 – 337 and other fragments at m/e 336, 330, 325, 320, 292, 278, 255, 240, 220.



Peak Numb	Time [min]	Type	Peak Width [min]	Area [Intens. * se	Height	Description	Start Point Time[min]	Start Point Height	End Point Time[min]	End Point Height
1	10.1	BB	0.0277	1402384.62	727564.02		10.0	6771	10.2	23922
2	10.4	BB	0.0305	317585.75	113547.67		10.4	22819	10.5	17136
3	12.4	BB	0.0347	773818.33	341351.44		12.3	1986	12.5	4601
4	13.1	BB	0.0390	2763567.20	952886.21		13.1	77734	13.3	15854
5	13.5	BB	0.0416	325933.72	125293.69		13.4	31264	13.5	13191
6	17.4	BB	0.0388	1098247.60	342259.63		17.4	3119	17.6	13934
7	20.3	BB	0.0342	587946.92	184797.08		20.2	5119	20.4	13774
8	23.8	BB	0.0478	451168.19	144918.05		23.7	4829	23.8	6435

Figure 5: Mass spectra of isolated compound

Interpretation of isolated compound:

Isolated compound gives positive test for alkaloid which indicate, the compound have alkaloid in nature. It is soluble in methanol having melting point 144-146°. Thin layer chromatography of isolated compound showed R_f value 0.65 using mobile phase butanol: acetic acid: water (6:1:3). UV spectra of isolated compound showed λ max at 430 nm. IR spectra of isolated compound also support the alkaloidal nature of the isolated compound. Bands at 3550 $cm^{-}1$, 2931 cm^{-1} and

1639 cm^{-1} indicates N-H, C-H and C=O stretching of the compound. PMR spectra of isolated compound also suggest peak at 7.27 ppm singlet indicates that the isolated compound is indole alkaloid in nature. Other values of PMR spectra also support the indole alkaloidal nature of the compound. Mass spectra of isolated compound showed molecular ion peak at m/e 336 which indicates molecular weight of the isolated compound was found to be 336.

Quantitative estimation of total phenolic contents (folin – ciocalteu method):

Standard Gallic acid was accurately weighed in volumetric flask and dissolved in 100 ml of distilled water and various concentrations of gallic acid (0.2-1.0 mg/ml) were prepared. From these various concentrations of gallic acid, 1ml was mixed with 5 ml of Folin- ciocalteu reagent (diluted tenfold) and then 4 ml of (7.5%) sodium carbonate. The absorption was measured after 30 m at 20° at 765 nm and the calibration curve was drawn. Methanolic extract (1 ml) was mixed with the same reagents as shown above, and after 30 min the absorption was measured at 765 nm for the determination of phenolic contents. Total phenolic content (%) in methanolic extract and isolated compound were calculated as (GAE) Gallic acid equivalent (Miniauskas, 2004). Standard calibration curve of gallic acid is shown in table 6 and figure 6. The results for total phenolic content in methanolic extract and isolated compound are shown in table 7.

$$GAE = [(C \times V) / M] \times 100$$

Where,

C = the conc. of Gallic acid established from calibration curve (mg/ml)

V = Volume of extract (ml)

M = the weight of dried plant extract (mg)

Table 6: Standard calibration curve of gallic acid

Sr. No.	Concentration (mg/ml)	Absorbance (765 nm)
1.	0.2	0.2140
2.	0.4	0.3998
3.	0.6	0.5001
4.	0.8	0.6101
5.	1.0	0.8044

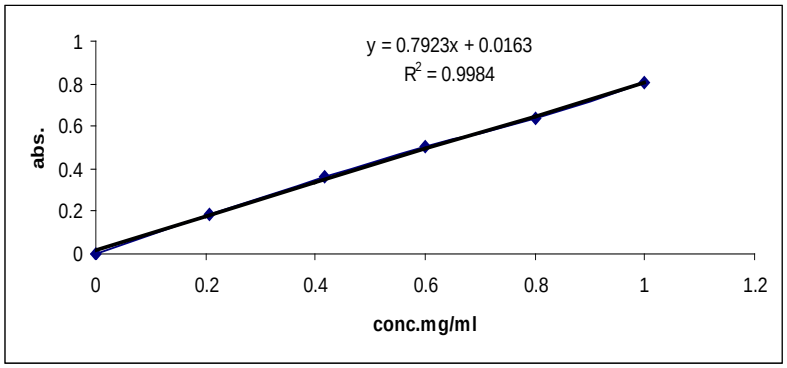


Figure 6: Standard calibration curve of Gallic acid

Table 7: Total phenolic content in methanolic extract and isolated compound

Sr .No.	Sample	Absorption at 765nm	GAE
1	Methanolic extract	0.6543	80.25
2	Isolated compound	0.6832	84.44

Evaluation of *in-vitro* antioxidant activity:

DPPH assay

Methanolic solution of isolated compound and methanolic extract were prepared at various concentrations (100, 200, 400, 800 and 1000 μg/ml). To a set of test tubes, 2.9 ml of DPPH solution (100 μg/ml in methanol) and 0.1 ml of varying concentrations of test sample were added. The mixture was then shaken vigorously and allowed it to stand in dark for 30 m and absorbance was measured using a UV spectrophotometer at 517 nm (Miliauskas, 2001). A control solution was consisting of 0.1 ml of methanol and 2.9 ml of DPPH radical solution (Desai and Wadekar, 2008). Percentage scavenging of DPPH radical was calculated by comparing the absorbance between the test, mixture and control.

Percentage scavenging of DPPH radical was shown in table 8 and figure 2.

$$\%\ \text{Scavenging of DPPH} = \frac{\text{Absorbance (control) - Absorbance (sample)}}{\text{Absorbance (Control)}} \times 100$$

Table 8: Results of percentage scavenging activity

Sr. No	Samples	Concentration µg/ml and % scavenging of DPPH				
		100	200	400	800	1000
1.	Methanolic extract	20.04	32.63	62.15	84.14	90.08
2.	Isolated compound	22.51	34.95	67.59	86.40	91.82
3.	Ascorbic acid	24.96	37.13	71.21	88.09	95.44

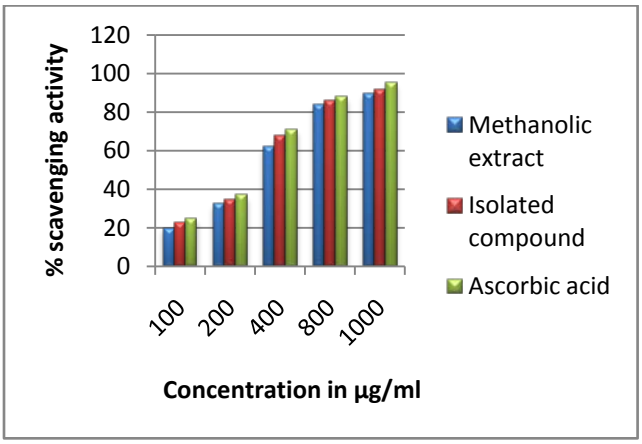


Figure 7: Percentage scavenging activity of DPPH

Reducing power method:

The reducing power of the extracts was carried out according to the conventional method. Various concentrations of methanolic extract and isolated compound (100, 200, 400, 800, 1000 µg/ ml) in 1.0 ml of deionized water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 1% of potassium ferricynide (2.5 ml). The mixture was incubated at 50^{o} for 20 m and aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was centrifuged at 1036 x g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared $Fecl_3$ solution (0.5 ml, 1%). The absorbance was measured at 700 nm (Scherer and Godoy, 2009; Gulcin *et al.*, 2004). Increased absorbance of the reaction mixture indicated increased reducing power. The results of reducing power of methanolic extract, isolated compound and ascorbic acid are shown in table 9 and figure 3.

Table 9. Reducing power of methanolic extract, isolated compound and ascorbic acid

Sr. No.	Sample	Concentration µg/ml and Absorbance at 700 nm				
		100	200	400	800	1000
1.	Methanolic extract	0.4899	0.8702	1.3114	2.0998	2.2148
4.	Isolated compound	0.6701	0.9065	1.7236	2.2054	2.7992
5.	Ascorbic acid	1.088	1.508	2.205	3.612	3.981

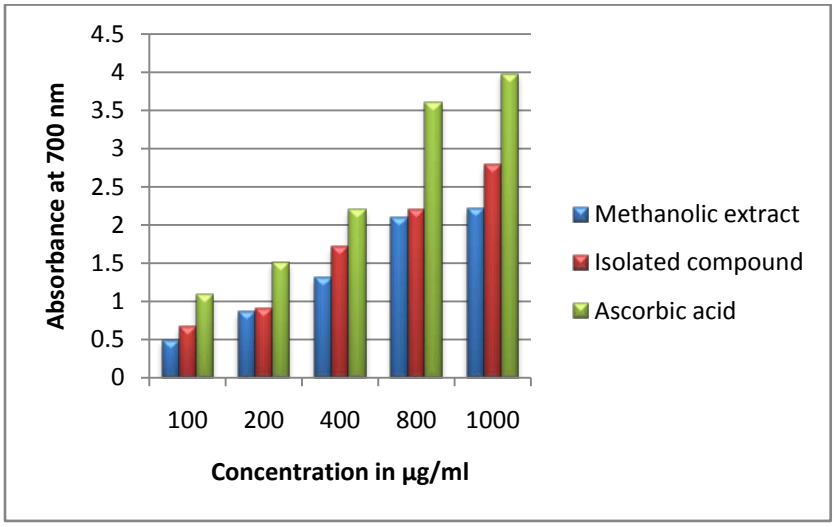


Figure 8: Reducing power of methanolic extract, isolated compound and ascorbic acid

DISCUSSION AND CONCLUSION

The present work deals with the study of extraction of *Albizia lebbeck*, its evaluation and validation for anti-proliferative activity. Bark was extracted with different organic solvents with increasing order of polarity namely petroleum ether, methanol and hydro alcoholic solution, respective extracts were obtained. Preliminary phytochemical screening showed that methanolic extract contain alkaloids and due to which extract was selected for isolation. Isolation of methanolic extract was carried out by column chromatography. Purity of isolated compound was checked by single spot on TLC. Isolated compound gave positive test for alkaloid (Dragendorffs test), indicating that isolated compound is alkaloid in nature. Isolated compound was screened for antioxidant activity by performing *invitro* assay method namely DPPH radical scavenging and reducing power method. Isolated compound showed higher antioxidant activity because it contains higher concentration of polyphenolic compound. The present study was designed to evaluate the *in-vitro* antioxidant potential of methanolic extract and isolated compound from *Albizia lebbeck* bark. The isolation of compound from methanolic extract was carried out by column chromatography technique and purity of isolated compound was checked by thin layer chromatography. The total phenolic contents of methanolic extract and isolated

compound were determined by using Folin - Ciocalteu method. The total phenolic content was high in the isolated compound (84.44 mg/g gallic acid equivalent GAE) as compared to methanolic extract (80.25 mg/g gallic acid equivalent GAE). The methanolic extract and isolated compound were investigated for free radical scavenging activity of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) and reducing power assay. The results of DPPH free radical scavenging at 1000 µg/ml indicated maximum antioxidant activity of 91.82% and 90.08% respectively. The reducing power of isolated compound and methanol extract were found to be 2.799 nm and 2.2148 nm respectively. The phytochemical screening revealed the presence of alkaloids and polyphenolic compounds. This suggest a potential utility of the plant as a source of phenolic antioxidants and may provide leads in the ongoing research for natural antioxidants form Indian medicinal plants to be used in treating diseases related to free radical reactions.

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