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Evaluation of free radical scavenging activity of *Butea monosperma* Lam.

Manish S Lavhale & S H Mishra *

Pharmacy Department, Faculty of Technology and Engineering, The M.S.University of Baroda, Vadodara 390 002, India

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In the present study, ethyl acetate, butanol and aqueous fractions derived from total methanol extract of *Butea monosperma* flowers were evaluated for radical scavenging activities using different *in vitro* models like reducing power assay, scavenging of 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide radical, superoxide anion radical, hydroxyl radical and inhibition of erythrocyte hemolysis using 2, 2′ azo-bis (amidinopropane) dihydrochloride (AAPH). Methanol extract along with its ethyl acetate and butanol fractions showed potent free radical scavenging activity, whereas aqueous fraction was found to be devoid of any radical scavenging properties. The observed activity could be due to the higher phenolic content in the extracts (16.1, 25.29, and 17.74%w/w in methanol extract, ethyl acetate and butanol fractions respectively). HPTLC fingerprint profile of the ethyl acetate and butanol fractions were developed which would serve as reference standard for quality control of the extracts.

Keywords: *Butea monosperma*, Free radicals, HPTLC fingerprint, Radical scavenging.

Reactive oxygen species (ROS) are generated continuously in the body by both endogenous and exogenous factors like normal aerobic respiration, by stimulated polymorpho-nuclear leukocytes, macrophages and exposure to various pollutants like tobacco smoke, ionizing radiation, organic solvents and pesticides[1,2]. Various reactive species includes the hydroxyl radical, superoxide anion, hydrogen peroxide, singlet oxygen, nitric oxide, hypochlorite, and various lipid peroxides[3]. These species cause the cellular damage by reacting with the various biomolecules such as membrane lipids, nucleic acids, proteins and enzymes. This damage is the major contributor for many disorders like cancer, hepatic ailments, cardiovascular diseases, cataracts, immune system decline, diabetes mellitus, inflammation, renal failure, brain dysfunction and the process of aging[4]. Antioxidants are the agents that are capable of effectively neutralizing the deleterious effects of free radicals. Natural antioxidants that are present in our body are catalase, superoxide dismutase, glutathione peroxidase, while synthetic antioxidants like butylated hydroxy toluene and butylated hydroxy anisole are suspected to be carcinogenic and hence are no more in use. Therefore, the need for the search of antioxidants from natural origin has been greatly felt in the recent years[5]. In Ayurvedic system of medicine, the flowers of *Butea monosperma* Lam (Leguminosae) commonly known as 'Flame of the forest', are used for relieving burning sensation, in treatment of gout, leprosy and other skin diseases[6]. In Unani system of medicine they are used as aphrodisiac, expectorant, tonic, emmenagogue, diuretic and in biliousness[7,8]. Chemical investigations showed that the flowers were found to contain butein, butin, butrin, isobutrin, coreopsin, isocoreopsin (butin 7-glucoside), sulphurein, monospermoside (butein 3-β -D-glucoside) and isomonospermoside. Bright colour of the flowers is attributed to the presence of chalcones and aurones. Earlier studies have shown that the plant possesses antifertility[9] antifungal[10], anthelmintic[11], hepatopro-tective[12], antiinflammatory[13], anti-diarrhoeal[14], anti-microbial[15], antiulcer and anticonvulsant properties[16]. It is evident that the plant has great potentials in treating a number of ailments where the free radicals have been reported to be the major factors contributing to the disorders. Thus, the present investigation was aimed to evaluate the antioxidant activity of total methanol extract of *B. monosperma* flowers along with its different fractions *viz.*, ethyl acetate, butanol and water, respectively.

Materials and Methods

Chemicals—2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′ azo-bis (amidinopropane) dihydrochloride (AAPH), nitroblue tetrazolium (NBT), 2-deoxy

*Correspondent author
Phone: +91 9427343031 / +91 265 2434187
Email: shmishra48@rediffmail.com

ribose, thiobarbituric acid (TBA), L-ascorbic acid, curcumin, quercetin, rutin and gallic acid, were procured from M/s Sigma Chemical Co. (St. Louis, USA). Other chemicals and reagents used were of analytical grade. UV-Visible spectrophotometer, Shimadzu 1700 was used for recording the spectra.

Plant material—Flowers of *Butea monosperma* Lam. were collected in the month of February-March from local area of Amravati, Maharashtra, India and were identified by comparing with the herbarium specimen. Voucher specimen (No. Pharmacy/05-06/BUT/ML) was submitted in the Pharmacy Department, The M S University of Baroda, Vadodara.

Preparation of extract—Coarse powder (200 g) of the shade dried flowers was extracted with methanol in a Soxhlet extractor, the extract was concentrated and dried under vacuum (yield 19.93% w/w).The vacuum dried extract (20 g) was suspended in water and then extracted with ethyl acetate and n-butanol (yield 1.16 g and 4.98 g, respectively). The remaining aqueous portion was concentrated and dried (yield 13.86 g). The total methanol extract along with its fractions were taken for further studies.

Preliminary phytochemical analysis

Methanol extract of *B. monosperma* flowers along with its fractions was subjected to qualitative phytochemical screening.

Evaluation of radical scavenging activity—Radical scavenging activity of methanol extract and its fractions was determined by measuring their reducing power, percentage inhibition of DPPH radical, superoxide radical, nitric oxide anion, hydroxyl radical and erythrocyte hemolysis induced by AAPH.

Determination of reducing power[17]—Reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Reductive ability was measured in terms of Fe^{3+}- Fe^{2+} transformation in the presence of different concentrations of the extract and fractions using the method of Oyaizu. Absorbance of the reaction mixture is directly proportional to the reducing power of the test substance. Ascorbic acid was used as a reference standard.

DPPH radical scavenging activity[18,19]—DPPH radical scavenging activity was determined according to the method of Blois[18] with some modifications. In brief, different concentrations of the test samples were incubated with 0.2 m*M* solution of DPPH for 30 min at room temperature and decrease in absorbance of the solution brought about by the sample was measured at 517 nm. Rutin was used as a reference standard.

Superoxide anion radical scavenging activity[20,21]—Superoxide radical scavenging activity was measured by riboflavin/light/NBT reduction method. The method was based on generation of superoxide radical by autooxidation of riboflavin in presence of light which in turn reduced NBT to a blue colored formazon. The capacity of extracts to inhibit the formazon formation was measured. Ascorbic acid was used as a reference standard.

Hydroxyl radical scavenging activity[22]—The method described by Halliwell was followed in the studies. It involved *in vitro* generation of hydroxyl radicals using Fe^{3+}/ascorbate/EDTA/H_2O_2 system by 'Fenton reaction'. Scavenging of hydroxyl radical in presence of different concentrations of test samples was measured at 532 nm. Curcumin was used as a reference standard.

Nitric oxide radical scavenging activity[23]—Nitric oxide was generated from sodium nitroprusside in aqueous solution under physiological *p*H and the extent of nitric oxide scavenged by the test samples was measured by the Greiss reaction. Curcumin was used as a reference standard.

Erythrocyte hemolysis[24,25]—AAPH, a water-soluble free radical generator was used to simulate the *in vivo* conditions of oxidative stress and to generate peroxyl radicals by thermal decomposition of an azo compound in oxygen. Addition of AAPH to the suspension of erythrocytes induces the oxidation of membrane lipids and proteins resulting in hemolysis. Inhibition of hemolysis in the presence of test sample was determined by measuring the absorbance of the supernatant fraction of reaction mixture at 540 nm. Ascorbic acid was used as a reference standard. In all the above methods, percentage inhibition was calculated using following formula—

% Radical scavenged =
[(Absorbance of control – Absorbance of test) x 100/ Absorbance of control]

where, control is the sample without test material and test is the sample containing the substance to be tested.

Rapid screening for antioxidant compounds[26]—To make a semi-quantitative visualization possible, total methanol extract of *B. monosperma* flowers along

with its ethyl acetate and butanol fractions were applied on a TLC plate (10 μg in methanol) and developed in a solvent system consisting of ethyl acetate: formic acid: glacial acetic acid: water (14.28:1.42:1.42:2.85 v/v/v/v). The plate was then dipped in a 0.2% solution of DPPH in methanol. The appearance of white and yellow colour spots on violet background was the indirect measure of radical scavenging components in the extract.

Determination of total phenolic content[27]—Total soluble phenolics in the extracts were determined according to the method of Shahidi and Wanasundara using Folin-Ciocalteau reagent, percentage of total phenolic content was calculated from calibration curve of gallic acid and was expressed as percentage equivalent to gallic acid.

TLC fingerprint profiles[28]—For HPTLC fingerprint profile, stock solution (1mg/ml) of the fractions was prepared in methanol. Different concentrations of samples were spotted on pre-coated Silica gel G60 F_{254} TLC plates using CAMAG Linomat V automatic sample spotter and the plates were developed in solvent systems of different polarities to resolve polar and non-polar components of the fractions. The plates were scanned using TLC Scanner 3 (CAMAG) at 254, 366 and 520nm. The R_f values, spectra, λ_{max} and peak areas of the resolved bands were recorded. Relative percentage area of each band was calculated from peak areas.

Results

Preliminary phytochemical analysis—Preliminary phytochemical studies revealed the presence of flavonoids and steroids in methanol extract and its ethyl acetate and butanol fractions, whereas aqueous fraction was found to contain only resinous mass.

Reducing power assay—Total methanol extract along with its ethyl acetate and butanol fractions were found to possess significant reducing power. Ethyl acetate fraction was more active compared to butanol fraction and total methanol extract. Aqueous fraction did not show any activity. The extracts exhibited a concentration dependent increase in reducing power. Compared with ascorbic acid the reducing powers of the three fractions were lesser (Fig. 1A).

Scavenging of DPPH radical—Total methanol extract along with its ethyl acetate and butanol fractions showed a concentration dependent antiradical activity by scavenging DPPH radical with an EC_{50} value of 22.88, 15.44 and 41.53 μg/ml, respectively. Ethyl acetate fraction was found to be more potent compared to butanol fraction and total methanol extract. Aqueous fraction did not show any inhibition. Compared with reference standard rutin (EC_{50}: 2.85 μg/ml; Fig. 1B), the scavenging effects were lesser.

Inhibition of superoxide radical—Ethyl acetate and butanol fractions were found to exhibit better inhibition of superoxide radical with an EC_{50} value of 4.03 and 18.49 μg/ml, respectively, while total methanol extract showed 50% inhibition at a higher concentration of 91.89 μg/ml. The aqueous fraction did not show any inhibition of superoxide radical. The results were compared with reference standard quercetin (EC_{50}:10.84 μg/ml; Fig. 1C). Ethyl acetate fraction was more effective than the standard, quercetin.

Inhibition of hydroxyl radical—Effect of methanol extract along with its ethyl acetate, butanol and aqueous fractions on deoxyribose damage induced by Fe^{3+}/H_2O_2 is shown in (Fig. 1D). The ethyl acetate fraction was found to show better inhibition compared to butanol fraction and methanol extract, with EC_{50} 15.45, 43.55 and 63.61 μg/ml, respectively. Aqueous fraction did not show any inhibition in deoxyribose damage. The results were compared with reference standard curcumin (EC_{50} 0.96 μg/ml), which was more effective than the fractions tested.

Inhibition of nitric oxide radical—Concentration of ethyl acetate, butanol fractions and total methanol extract required for 50% inhibition were found to be 225.66, 66.20 and 281.74 μg/ml respectively. Butanol fraction was found to be more potent compared to methanol extract and its ethyl acetate fraction. Aqueous fraction did not show any inhibition of nitric oxide. Curcumin which was used as a reference compound, showed 50% inhibition at 34.23 μg/ml (Fig. 1E).

Inhibition of erythrocyte hemolysis—The methanol extract along with its ethyl acetate and butanol fractions were found to inhibit the hemolysis of erythrocytes. Butanol and ethyl acetate fractions were found to be more potent compared to total methanol extract with EC_{50} values of 2.26, 8.35 and 11.41 μg/ml, respectively. The results are comparable to that of ascorbic acid (EC_{50} 3.07 μg/ml). The butanol fraction was found to be more potent than the ascorbic acid. Aqueous fraction did not show any inhibition in AAPH induced erythrocytes hemolysis (Fig. 1F).

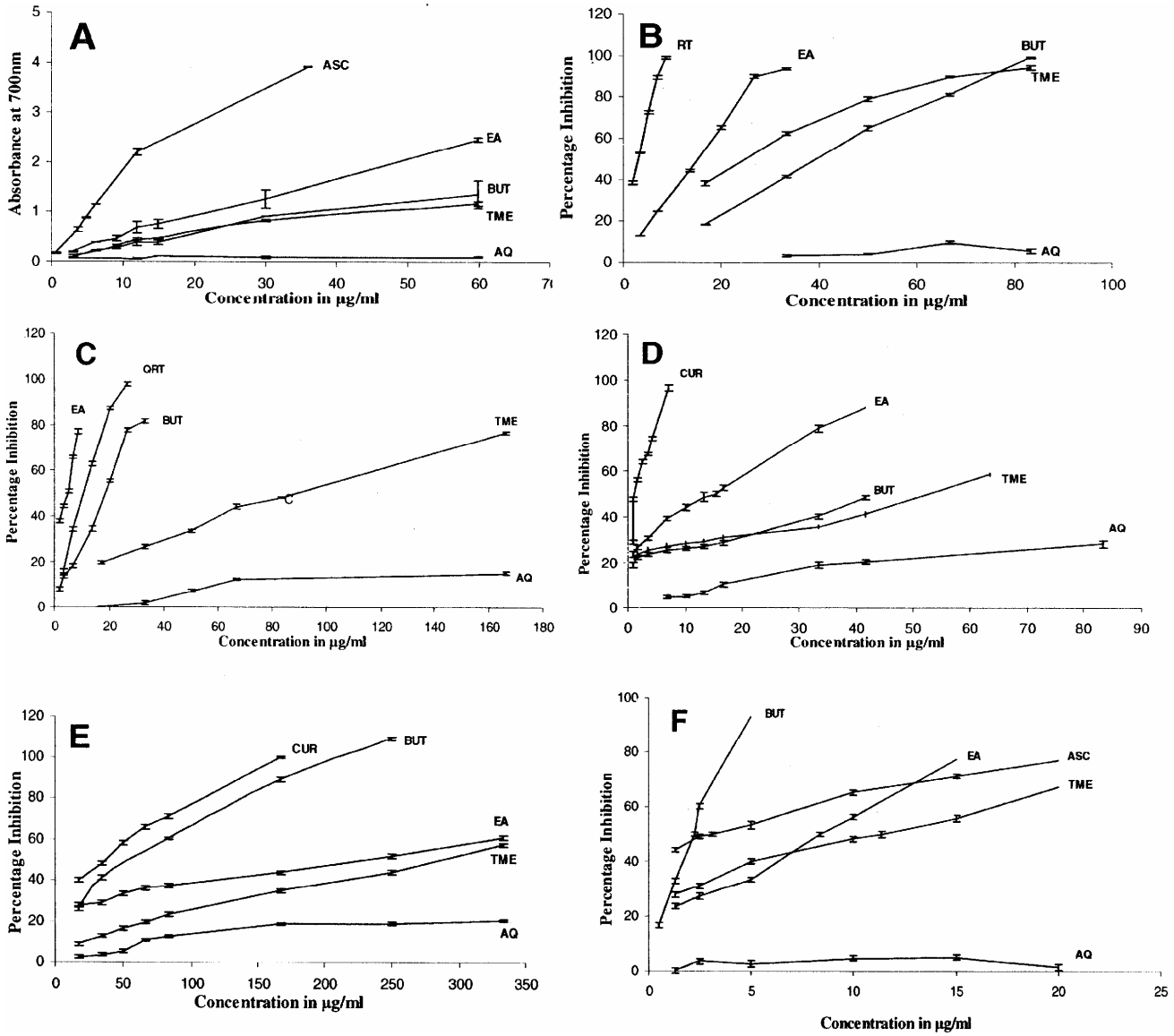

Fig. 1—Free radical scavenging activity of total methanol extract of *Butea monosperma* flowers and its fractions (A) Reducing power; (B) Scavenging of DPPH radical; (C) Inhibition of superoxide radical; (D) Inhibition of hydroxyl radical; (E) Inhibition of nitric oxide radical; and (F) Inhibition of erythrocyte hemolysis. [Absorbance values are Mean ± SD of triplicates. EA- Ethyl acetate fraction; BUT- Butanol fraction; TME- Total methanolic extract; AQ- Aqueous fraction; ASC-Ascorbic acid; RT-Rutin; QRT-Quercetin; and CUR-Curcumin].

Rapid screening for antioxidant compounds—Total methanol extract and its fractions *viz.*, ethyl acetate and butanol were found to contain antiradical components with ethyl acetate fraction containing higher concentration than butanol fraction and methanol extract (Fig. 2).

Total phenolic content—The total amount of phenolic content present in total methanol extract and its ethyl acetate, butanol and aqueous fractions was found to be 16.1, 25.29, 17.74 and 0.9% w/w, respectively.

TLC fingerprint profile—TLC fingerprint profile of the ethyl acetate and butanol fractions were recorded. (Tables 1, 2 and Figs 3, 4)

Discussion

Interest in the search for natural antioxidants has increased over the past few years as the reactive oxygen species (ROS) production and oxidative stress have been shown to play vital role in a number of disorders[29]. Also the restrictions laid on the use of synthetic antioxidants have been the important

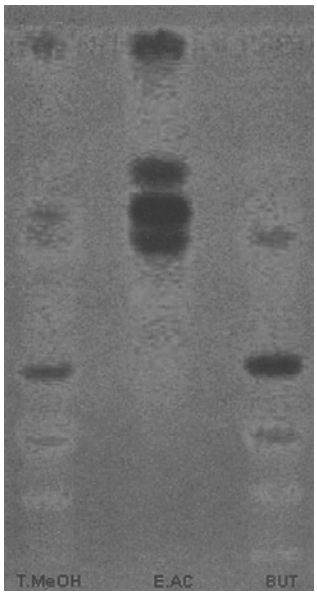

Fig. 2—Rapid screening of Butea total methanol extract and its fractions for antioxidant activity using pre-coated Silica gel G60 F_{254} TLC plates, dipped in DPPH reagent. The complexes formed shows yellow and white coloration on the TLC plate. [T.MeOH-Total methanol extract; E.AC-Ethyl acetate fraction; and BUT-Butanol fraction].

incentive for such research work. These studies are more pertinent with regard to therapeutic agents of plant origin employed in treating a wide range of diseases[30]. Flowers of *B. monosperma* are reported to be used in many ailments including liver disorders. From the results it was observed that the methanol extract of the *B. monosperma* and its various fractions were found to act as radical scavengers against different free radicals under the conditions of oxidative stress. Most nonenzymatic antioxidative activity like scavenging of free radicals, inhibition of lipid peroxidation, etc. is mediated by redox reactions. The reducing power determined in the present study depends on the redox potentials of the compounds present in different fractions. The highest amount of reducing power was observed in the ethyl acetate and butanol fractions followed by total methanol extract. Thus, it can be expected that the fractions may have scavenging activity against other oxidizing agents. DPPH radical has been widely used to test the radical scavenging ability of various natural products and has been accepted as a model compound for free radicals originating in lipids[31]. Ethyl acetate and butanol fractions along with total methanolic extract showed a

Table 1—TLC fingerprinting for butanol fraction of *B. monosperma* flowers

Scanning wavelength	Solvent system 1*			Solvent system 2**		
	Rf	λ_{max}	Relative % area	Rf	λ_{max}	Relative % area
254nm (Under UV)	0.13	366	2.01	0.07	200	5.87
	0.33	277	44.78	0.09	200	3.08
	0.52	370	40.51	0.14	278	47.33
	0.93	415	6.74	0.16	374	43.72
366nm (Under UV)	0.29	276	11.42	0.11	200	49.52
	0.45	406	27.31	0.14	278	23.43
	0.59	406	18.86	0.17	200	12.22
	0.76	586	17.43	0.18	200	14.84
	0.91	414	24.98			
520nm (After Derivatization)	0.11	480	1.52	0.57	476	86.13
	0.26	480	20.85	0.65	474	13.87
	0.39	558	31.04			
	0.60	480	8.90			
	0.68	525	10.25			
	0.77	468	15.10			
	0.84	523	11.22			
	0.94	525	1.13			

*Solvent system 1—ethyl acetate: formic acid: glacial acetic acid: water (14.28:1.42:1.42:2.85 v/v) and treated with DPPH; **Solvent system 2—butanol: acetic acid: water (4:1:5 v/v), treated with anisaldehyde sulphuric acid reagent.

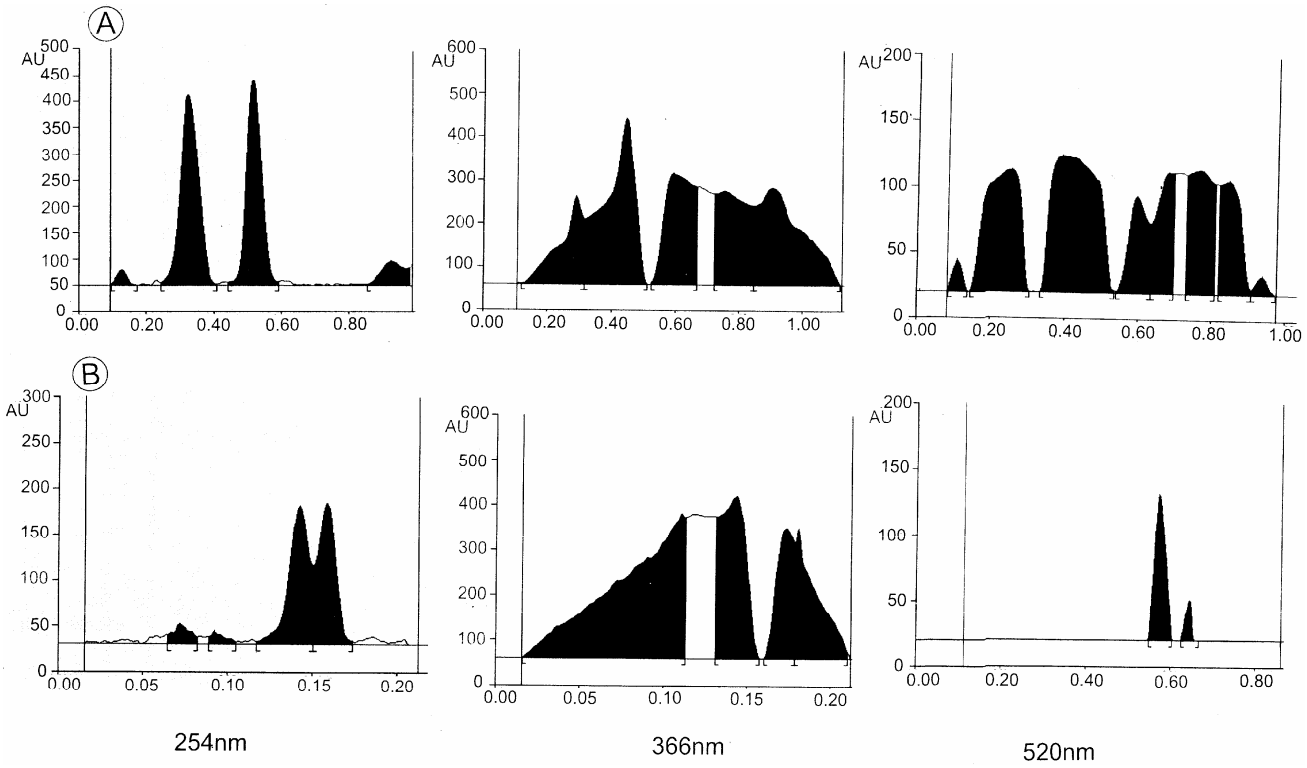

Fig. 3—TLC chromatogram of butanol fraction of *B. monosperma* flowers in two different solvent systems and scanned at 254, 366 and 520 nm A: Solvent system 1 and B: Solvent system 2.

Table 2—TLC fingerprinting for ethyl acetate fraction of *B monosperma* flowers

Scanning wavelength	Solvent system 1*			Solvent system 2**		
	Rf	λ_{max}	Relative % area	Rf	λ_{max}	Relative % area
254nm (Under UV)	0.53	374	1.48	0.04	278	36.46
	0.76	370	6.25	0.68	283	13.40
	0.93	284	57.41	0.75	345	19.25
				0.94	280	30.89
366nm (Under UV)	0.48	406	18.27	0.03	700	0.14
	0.63	373	13.65	0.17	700	15.13
	0.76	372	24.23	0.20	700	48.96
	0.92	278	37.71	0.62	681	12.34
				0.85	682	7.24
				0.95	700	7.36
520nm (After Derivatization)	0.26	420	19.79	0.05	100	371
	0.38	540	22.53			
	0.54	480	34.25			
	0.78	480	5.75			
	0.92	535	17.68			

*Solvent system 1: ethyl acetate: formic acid: glacial acetic acid: water (14.28:1.42:1.42:2.85 v/v) and treated with DPPH; **Solvent system 2: chloroform: acetone: formic acid (14.85:3.36:1.78 v/v) and treated with anisaldehyde sulphuric acid reagent.

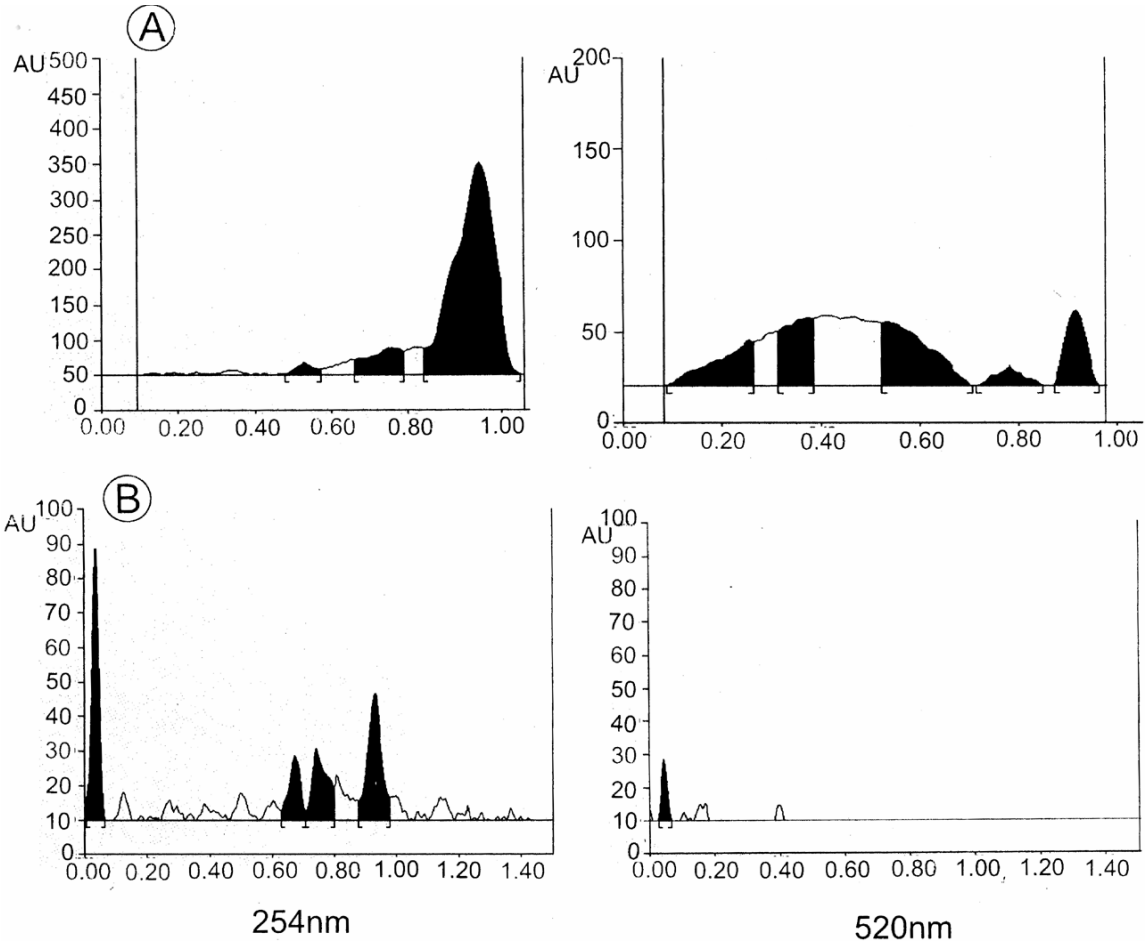

Fig. 4—TLC chromatogram of ethyl acetate fraction of *B. monosperma* flowers in two different solvent systems and scanned at 254 and 520 nm. A: Solvent system 1 and B: Solvent system 2

concentration dependent antiradical activity by reducing DPPH radical. Superoxide anion, which is a reduced form of molecular oxygen, has been implicated in initiating oxidation reactions associated with aging[32]. It plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA[33]. Ethyl acetate and butanol fractions were found to be good scavenger of superoxide radicals. The decrease of absorbance in the presence of these fractions indicates the consumption of superoxide anion in the reaction mixture. Hydroxyl radicals are reported to cause oxidative damage to various biomolecules like DNA, lipids and proteins[34]. Ethyl acetate and butanol fractions were capable of reducing the damage to deoxyribose more effectively than the total methanol extract. Sodium nitroprusside serves as a main source of nitric oxide radicals. Butanol and ethyl acetate fractions, scavenges the NO formed from the sodium nitroprusside by inhibiting the chromophore formation and hence the absorbance decreases as the concentration of the fractions increases.

The present study was further supported by the finding that the ethyl acetate and butanol fractions strongly inhibited erythrocyte hemolysis induced by AAPH, where lysis occurs mainly by two events, lipid peroxidation and redistribution of oxidized band 3 within the cell membrane. It is considered that AAPH attack the membrane to induce the chain oxidation of lipids and proteins leading to the damage of spectrin, a membrane protein, by oxidative cross linking which may ultimately result in hemolysis. Band 3 in erythrocyte membranes plays an important role in a rapid exchange of $HC03^-$ and $C1^-$ across the membrane[35]. Butea fractions may block the hemolysis by inhibiting the formation of hemolytic holes in the

erythrocytes cell membrane by blocking oxidation and redistribution of band 3 proteins[36]. Preliminary phytochemical investigation of ethyl acetate and butanol fractions showed the presence of phenolics including flavonoids as a major class of components. Further, the butanol fraction of the total methanol extract has been reported to contain butrin and isobutrin, the antihepatotoxic agents[37]. HPTLC fingerprinting profile of ethyl acetate and butanol fractions was generated in solvent systems of different polarities in order to ascertain the total number of chemical moieties which will also help in designing the method of isolation and characterization of the bioactive components.

Rapid screening for antioxidants by TLC revealed the presence of many constituents with radical scavenging properties in varying proportions in all the fractions other than aqueous. These constituents can be isolated and can be used for future structural elucidation. These studies suggested that specific constituents in the ethyl acetate and butanol fractions contribute to the antioxidant activity of the flowers. Phenolic compounds are reported to be potent free radical terminators and thus, the results are further supported by the varying amounts of total phenolic content in different fractions of flowers[38].

In conclusion, in the present study, ethyl acetate and butanol fractions were found to have potent free radical scavenging activity and can also act by inhibiting the AAPH mediated erythrocyte hemolysis. The observed activity may be mainly due to their total phenol content. However, in case of erythrocyte hemolysis, butanol fraction with lower phenolic content was found to be more effective than ethyl acetate fraction with higher phenolic content, which may be due to the presence of two antihepatotoxic principles *viz*., butrin and isobutrin. Further investigation on the isolation of the constituents and *in vivo* studies are necessary to utilize the antioxidant potentials of the plant and the steps in this regard are already being undertaken by the authors.

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