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Antioxidant Potential Profile of Extracts from Different Parts of Black Mulberry

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Present study is aimed to exploit a potent and cheaper natural sources of antioxidants. *Morus nigra* L. is considered to be an antidiabetic and diuretic agent in the folk medicinal system of Indo-Pak subcontinent. Antioxidant potential and radical scavenging activity of the various organic and aqueous extracts of leaves, stem and fruit of *M. nigra* were investigated using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) decolourization assay, ferric reducing antioxidant power (FRAP) assay, 2.2'-diphenyl-1-picryl-hydrazyl (DPPH) assay, total phenolic content (TPC) assay and total antioxidant activity assay. Using $ABTS^{\bullet+}$ decolourization assay and FRAP assay, *M. nigra* extracts showed a wide range of antioxidant activity. Using TPC assay the amount of total phenolics ranged from 1.3048 to 5.8287, 0.413 to 2.9394 and 0.5346 to 6.2266 mg/L for different fractions of leaves, stem and fruit of *M. nigra*, respectively. Good co-relation was found between TPCs and TEAC values determined by $ABTS^{\bullet+}$ decolourization assay (r^2 = 0.977, 0.943 and 0.823 for leaves, stem and the fruit of stem and FRAP assay (r^2 = 0.887, 0.842 and 0.773 for leaves, stem and the fruit) of *M. nigra*. Employing total antioxidant assay using linoleic acid emulsion system, methanol, 1-butanol, aqueous and ethyl acetate fractions of all the parts of *M. nigra* showed strong peroxyl radicals scavenging activity. The data obtained by employing anti-oxidant and radical scavenging assays demonstrated the effectiveness of all the parts of *M. nigra* as antioxidative and radical scavenging which may also be correlated with its anti-disease activity in *in vivo*.

Key Words: Antioxidant potential, Radical scavenging, TEAC, *Morus nigra*.

INTRODUCTION

The oxidative stress, which is basically an imbalance between oxidants and antioxidants in favour of the oxidants, has been suggested to be the cause of various degenerative diseases or the complications arising after the occurrence of these diseases. Due to health-promoting effects of antioxidants a general recommendation to the consumer is to increase the intake of foods rich in antioxidant compounds (*e.g.* polyphenols, flavonoids)[1-12]. Phenolic compounds, especially flavonoids and anthocyanins, are very important antioxidants because of their natural origin and their ability to act as efficient free radical scavengers[12-15]. Small berries have been

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reported to be rich sources of phenolic compounds such as phenolic acids as well as anthocyanins, proanthocyanidins and other flavonoids, which display potential health promoting effects[16-24].

Morus nigra L. (black mulberry) is regarded as a diuretic and antidiabetic, laxative, antidiarrheal, antiinflammatory, antirheumatic, antitussive[25-27]. Stem tissues of *M. nigra* is shown to have significant antioxidant activity because of phenolic and anthocyanin constituents[28]. Major phenolic acids in *M. nigra* have been reported to be 1-gallic, 2-gentisic, 3-protocatechuic, 4-salicylic, 5-*p*-coumaric and 6-ferulic[29]. The major fatty acids in mulberry fruits are shown to be linoleic acid (54.2 %), palmitic acid (19.8 %) and oleic acid (8.41 %)[30]. Two major anthocyanins of *Morus nigra*, isolated by paper chromatography, have been reported to be cyanidin-3-glucoside and cyanidin-3-glucosylrhamnoside, with no cinnamoyl groups[31].

Antioxidant flavonoids of *Morus nigra* L. (Moraceae) have been analyzed by capillary electrophoresis. Quercetin-3-O-β-rutinoside (rutin), quercetin-3-O-β-D-glucoside (isoquercitrin) and chlorogenic acid are reported to be the most abundant compounds in *Morus nigra*[32]. Hyperglycaemia in diabetes mellitus is responsible for the development of oxidative stress through increased lipid peroxide production[33].

Although, a lot of discussion regarding antioxidant potential of *M. nigra* has been made in the literature, yet no comparative profile of radical scavenging and reducing activity of various extracts from different parts of this plant has ever been attempted. The main objective of this study is to investigate and compare the antioxidative activity of various extracts of stem (MS), leaves (ML) and fruit (MF) of *M. nigra*. Total antioxidant potential has been determined using ferric reducing/ antioxidant power assay (FRAP)[34]. Total phenolic contents were determined according to Folin-Ciocalteu colorimetric method[35]. The free radical scavenging activity of *M. nigra* was determined using two different stable free radicals DPPH radical and ABTS radical.

EXPERIMENTAL

All the chemicals used were of analytical grade. Methanol, 1-butanol, chloroform, *n*-hexane, ethyl acetate, glacial acetic acid, potassium persulphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, iron(III) chloride, iron(II) chloride were purchased from E. Merck while trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), ABTS (2,2'-azinobis-(3-ethylbenzo-thiazoline)-6-sulphonic diammonium salt), gallic acid, Tween 20 (polyoxyethylene-sorbitan monolaurate), iron(II) sulphate, potassium thiocyanate, 1,1-diphenyl-2-picryl-hydrazil (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteau's reagent (FC Reagent) were purchased from Fluka (Switzerland).

Plant material: Different parts (stem, leaves and fruit) of *M. nigra* growing abundantly in Indo-Pak subcontinent, were collected from Botanic Garden, GC University, Lahore, Pakistan. Freshly collected plant materials were cleaned with

distilled water to remove adhering dust particles and then dried under shade. The dried samples were pulverized in a Willy Mill to 60-mesh size and used for solvent extraction.

Solvent extraction: Air-dried ground plant samples of stem (MS), leaves (ML) and fruit (MF) were extracted individually with cold methanol (1:20 w/v) in cork-fitted flasks for 24 h at 30 ºC and 240 rpm. The extraction was repeated three times to ensure complete extraction and the solvent was evaporated under vacuum conditions. The methanolic extracts of different parts were suspended separately in 10 volumes of water and then partitioned successively with *n*-hexane, chloroform, ethyl acetate and *n*-butanol (25 × 3 mL), leaving a residual water-soluble fraction. The extracts thus obtained were stored at 4 ºC until they were used for the estimation of total phenolics and the antioxidant potential. For each plant sample, three replicates of the extracts were prepared.

Total phenolic content assay: Total soluble phenolics in *M. nigra* plant extracts were determined with Folin-Ciocalteu's reagent according to the method of Singleton *et al.*[35] using gallic acid as a standard phenolic compound. A volume of 40 µL of each sample and standard was transferred into separate test tubes and to each added 3.16 mL water and 200 µL of Folin-Ciocalteu's reagent. The mixture was mixed well, kept for 8 min and then added 600 µL of sodium carbonate solution with continuous stirring. The solution was left at 40 ºC for 0.5 h and absorbance of each solution at 765 nm against the blank (without phenolic solution) was determined. A curve was plotted between absorbance and concentration. The concentration of total phenolic compounds of all fractions of *Morus nigra* plants was determined as milligrams of gallic acid equivalent (GAE) by using the following equation that was obtained from the standard gallic acid graph.

$$\text{Absorbance} = 0.0555 \times \text{Gallic acid (mg/L)}$$

Ferric reducing antioxidant power (FRAP) assay: FRAP assay was performed as described by Benzie *et al.*[34]. Experiment was conducted at 37 ºC at pH 3.6 with a blank sample. FRAP reagent was prepared by mixing 25 mL acetate buffer (pH 3.6), 2.5 mL (10 mmol/L) TPTZ solution and 2.5 mL (20 mmol/L) $FeCl_3 \cdot 6H_2O$ solution. 300 µL freshly prepared FRAP reagent was warmed to 37 ºC and a reagent blank reading was taken at 593 nm; 10 µL of sample/standard antioxidant was then added along with 30 µL H_2O. Absorbance readings were taken after every 1 min for 4 min. The change in absorbance (ΔA_{593}) between final reading and reagent blank was noted. For reference data ΔA_{593} values for a range of trolox concentrations were noted and a standard curve was drawn. Final results were expressed as micro-mole trolox equivalents (TE) per gram on dried basis (µmol TE\g, db).

$ABTS^{\bullet+}$ Decolourization assay: $ABTS^{\bullet+}$ Assay protocol as developed by Re *et al.*[36] was followed. ABTS was dissolved in doubly distilled water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to

stand in the dark at room temperature for 12-16 h before use. For the study of antioxidant activity of standard antioxidant and plant samples, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.700 (± 0.020) at 734 nm and equilibrated at 30 ºC. For plant extracts, the dilution was made in the respective solvents. After addition of 10 µL of neat or diluted stock solution to 2.99 mL of diluted $ABTS^{\bullet+}$ solution (A = 0.700 ± 0.020), the absorbance reading was taken at 30 °C exactly 1 min after initial mixing up to 8 min. Appropriate solvent blanks were run in each assay. The percentage inhibition of absorbance was calculated by the following formula and was plotted as a function of concentration of antioxidants and of Trolox for the standard reference data

$$\% \text{ Inhibition (at 734 nm)} = (1 - A_f / A_o) \times 100$$

where A_o is the absorbance of radical cation solution before addition of sample/standard antioxidants while A_f is the absorbance after addition of the sample/standard antioxidants. Each measurement was made in triplicate and at least three times at each concentration level of standards and sample.

2,2'-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH Assay): Free radical scavenging activity of water and organic solvent extracts was measured by 2,2'-diphenyl-1-picryl-hydrazil ($DPPH^{\bullet+}$) using the method of Shimada *et al.*[37] DPPH solution (3 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions. The reaction progress of the mixture was monitored at 515 nm over a time period. Upon reduction, the colour of the solution faded. The percentage of the DPPH remaining was calculated as

$$\% \text{ DPPH}_{rem} = [\text{DPPH}]_{rem} / [\text{DPPH}]_{t=0} \times 100$$

where $[DPPH]_{rem}$ corresplnds to absorbance of DPPH solution at time t while $[DPPH]_{t=0}$ is the absorbance before the addition of sample or standard antioxidant.

A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 593 nm as a function of time (min) was plotted for each fraction of the samples.

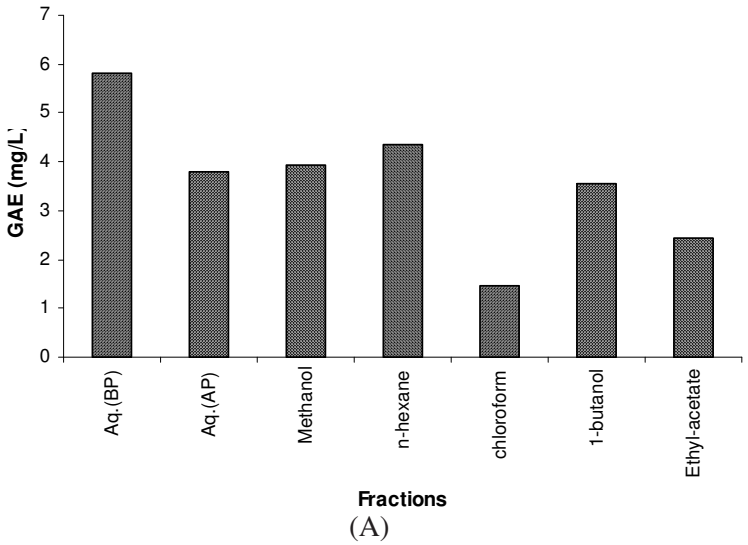
Total antioxidant activity determination: Total antioxidant activity of aqueous and organic extracts of *Morus nigra* plant was determined according to the method employed by Mitsuda *et al.*[38]. The solution, which contain 100 µL each of neat or diluted plant extract of *Morus nigra* plant in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Each solution was then incubated at 37 ºC in sealed bottles in dark. The solution without added extract was used as blank, while the solutions containing 100 µL (50 mg/20 µL) of Trolox was used as positive control. At intervals of 24 h during incubation, 0.1 mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1 mL each of $FeCl_2$ (20 mM in 3.5 % HCl) and thiocyanate solution (30 %) to the ethanolic sample, the solution was stirred for 1 min. The absorption values of the solutions measured at 500 nm were taken as lipid peroxidation values.

RESULTS AND DISCUSSION

Total phenolic contents and its correlation with Trolox equivalent antioxidant capacity: Hydroxyl radical produced *in vivo* is considered as the most devastating amongst reactive oxygen species. ABTS radical cation has the same reduction potential to that of hydroxyl radical and may be taken as equivalent to $OH^{\bullet}$ in *in vitro* test environment. ABTS radical scavenging ability and ferric reducing ability of the extracts of various part of *M. nigra* were evaluated using ABTS radical cation decolourization assay and FRAP assay, respectively.

Total phenolic contents in terms of gallic acid equivalents of all the extracts were determined using Folin-Ciocalteu's method. The extracts showed high GAE values (Fig. 1). These values may be attributed to the presence of various phenolic acids and flavonoid content[29-31]. The amount of total phenolics ranged from 1.3048 to 5.8287, 0.4134 to 2.9394 and 0.5346 to 6.2266 mg/L for different fractions of leaves, stem and furit of *M. nigra*, respectively. Aqueous (BP) fractions of leaves (5.8287 mg/L), stem (2.9272 mg/L) and fruit (0.9325 mg/L), methanolic fractions of leaves (3.9492 mg/L), stem (2.6972 mg/L) and fruit (2.1435 mg/L) contained the highest amounts of phenolic compounds. Significant amount of phenolic compounds were also found in 1-butanol fractions of all the three parts while chloroform and *n*-hexane were found to be containing least amounts of phenolic content.

Correlations between total phenolic content of various extracts of *M. nigra* and ABTS radical cation scavenging activity and the ferric reducing ability of the corresponding fractions have been shown in Fig. 2. The statistical analysis showed a positive and highly significant relationship (r^2 = 0.887, 0.842, 0.773 and 0.977, 0.943, 0.823 for TPC assay *vs.* FRAP assay and TPC assay *vs.* ABTS radical cation decolourization assay, respectively) between total phenolic contents and antioxidant activity. It is also evident from the data that ABTS radical cation decolourization



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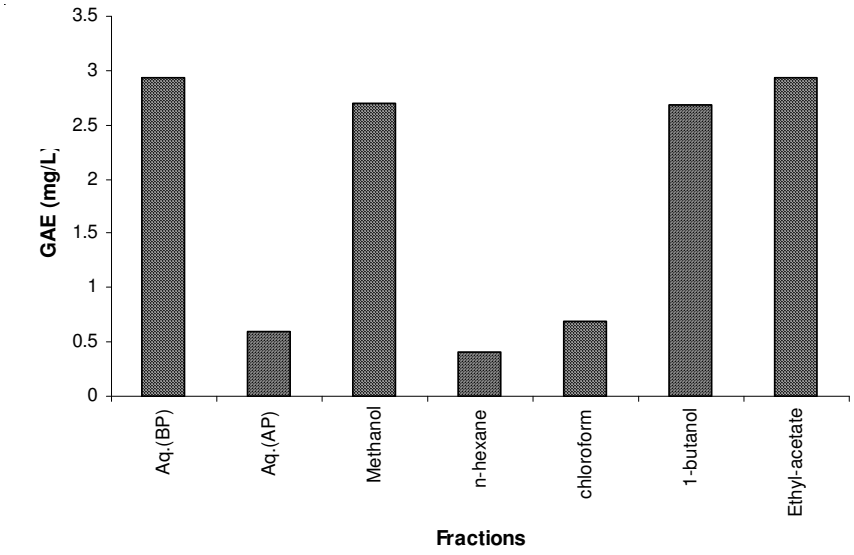

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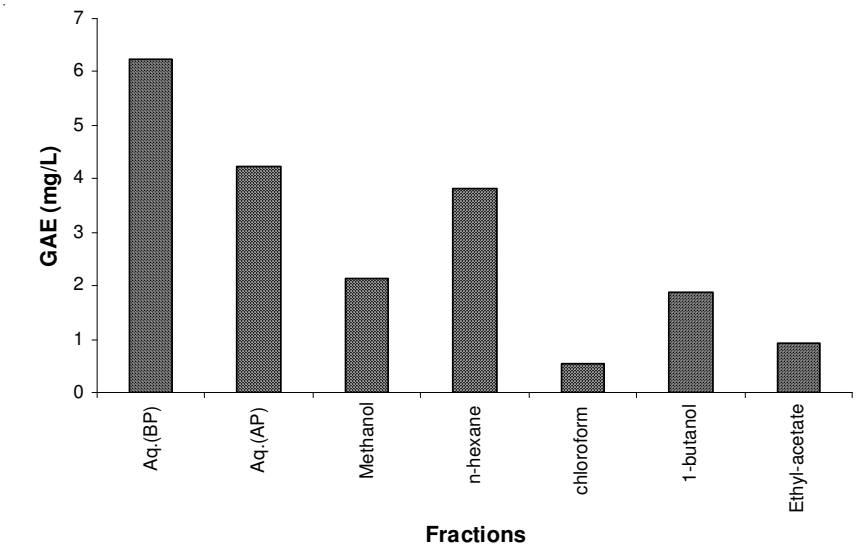
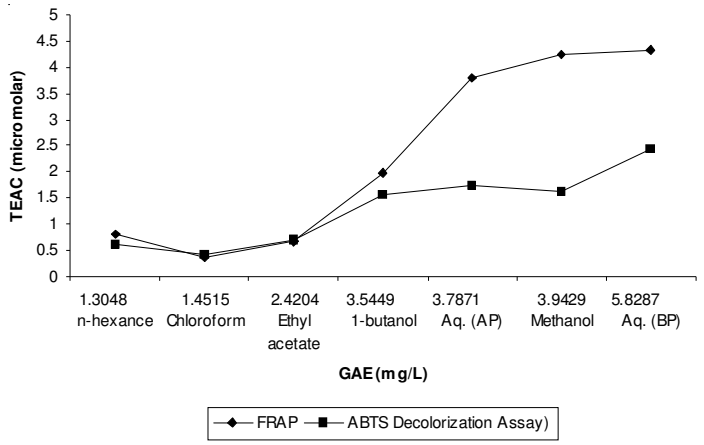


(C)

Fig. 1. Comparison of GAE values of various extracts of (A) leaves (B) and stem (C) pulp of *M. nigra* using TPC assay

assay is more linearly related to TPC as compared with FRAP assay. Attempts have been made to derive a relationship between the phenolic contents and antioxidant activity. Controversial results have been obtained regarding a linear relationship between TPC and antioxidant activity[39-41].



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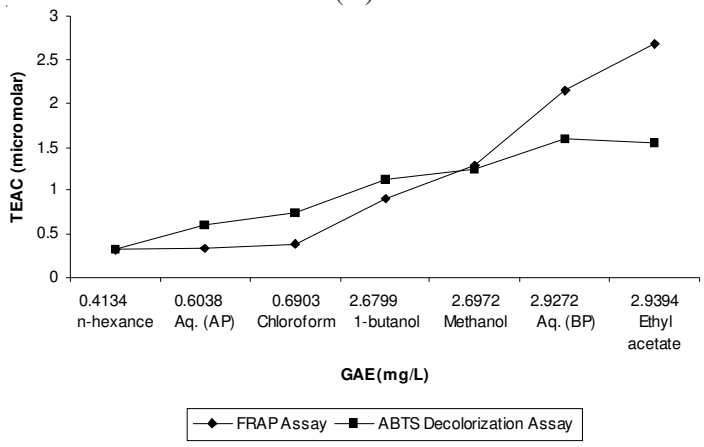

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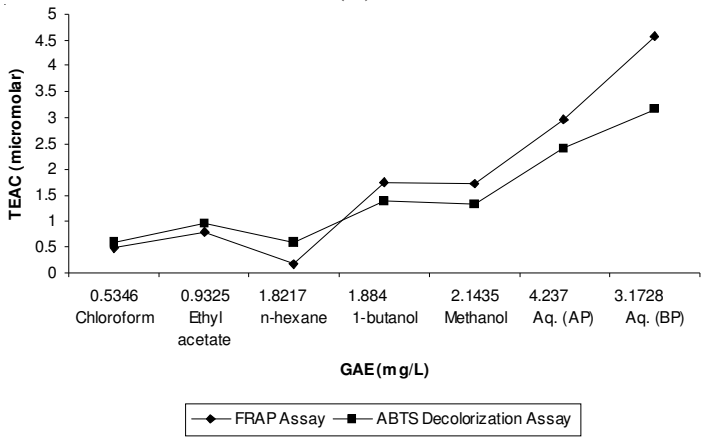


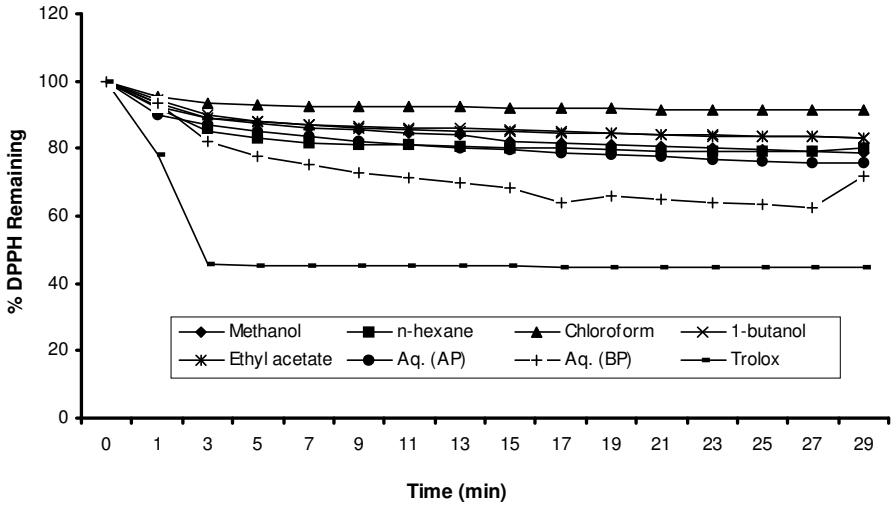
(C)

Fig. 2. Correlation between TEAC values (obtained from FRAP assay and ABTS decolorization assay) and GAE values (obtained from TPC method)

The present study showed a relatively good relationship between TPC and antioxidant activity determined through ABTS radical cation decolourization assay and FRAP Assay. Non-acquisition of absolutely linear relationship between TPC and the two assays may be due to different response of different phenolics in Folin-Ciocalteau reagent[42,43] difference in the pH of the medium of assays and the reduction potential of the oxidized species. Furthermore the antioxidant activity strongly depends upon the chemical structure of phenolic compounds. Therefore no definite quantitative relationship could be obtained for general application to all the plant extracts.

DPPH and lipid peroxy radicals scavenging activity: It is well established that antioxidants inhibit free radical chain of oxidation. DPPH and lipid peroxide free radicals have been used to evaluate reducing properties and to assess free radicals chain breaking abilities of phyto-chemicals. Fig. 3 summarizes the kinetics and scavenging activities of DPPH radicals by various extracts from different parts of *M. nigra*. The kinetics of the scavenging of DPPH free radicals showed that almost all the extracts from three parts of *M. nigra* had appreciable scavenging effect which was quite rapid in the first three minutes of mixing and later became slow and gradual. This behaviour showed the presence of fast-reacting and slow reacting antioxidants in the extracts. It could be inferred from the figures that majority of the components were fast-reacting because after three minutes of mixing there was a very slight decrease in the % DPPH remaining. Aq. (BP) fractions of leaves and fruit while ethyl acetate fraction of stem showed scavenging activities comparable to that of trolox.

In biosystems, unsaturated fatty acids are always at stake due to free radicals attack on the bio-membranes which results in membrane lipid peroxidation, decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane



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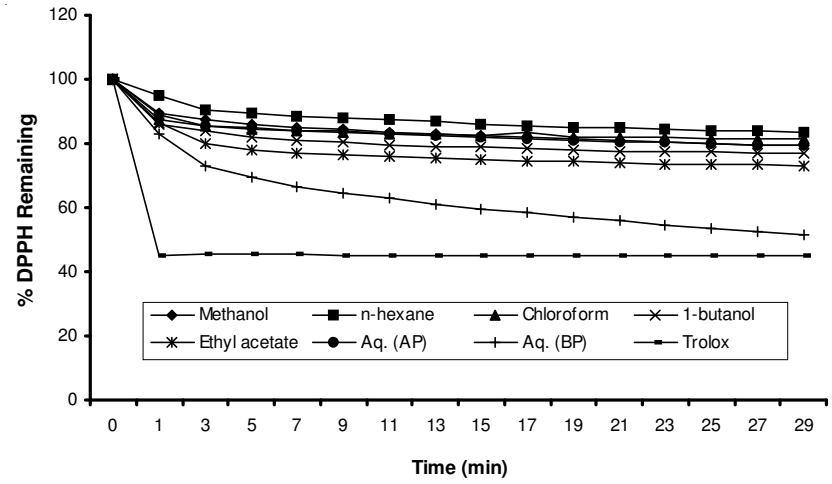

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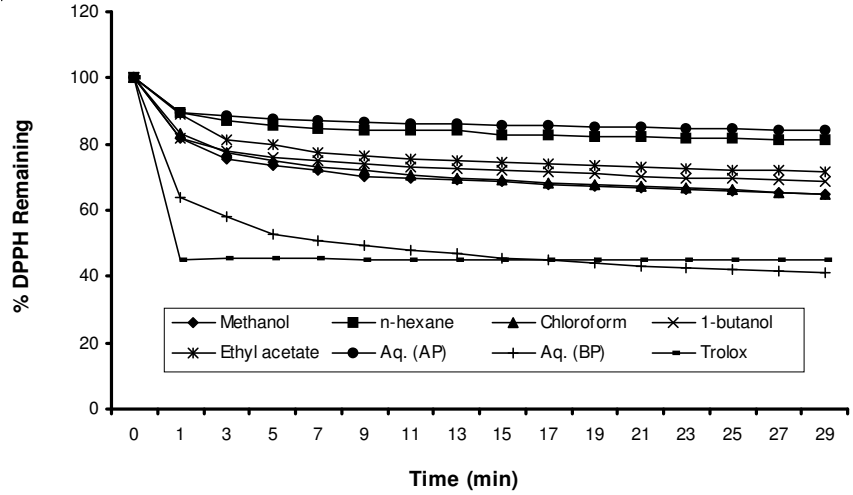
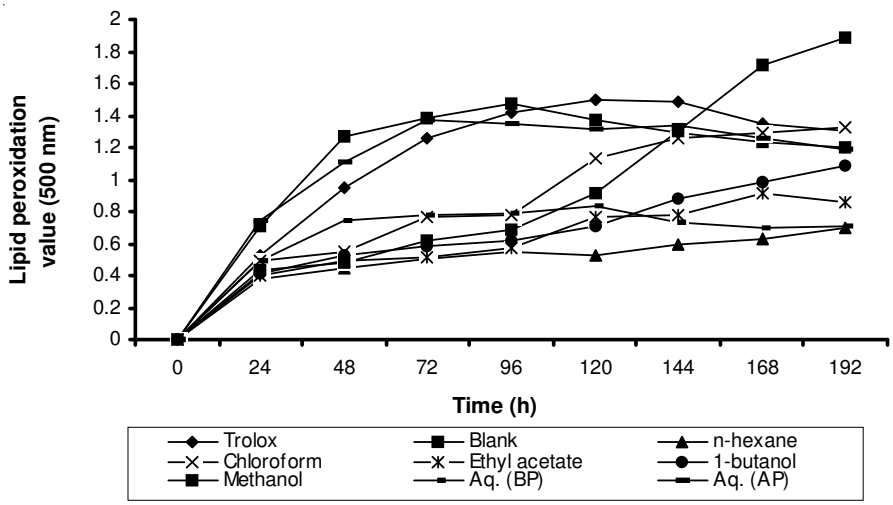


(C)

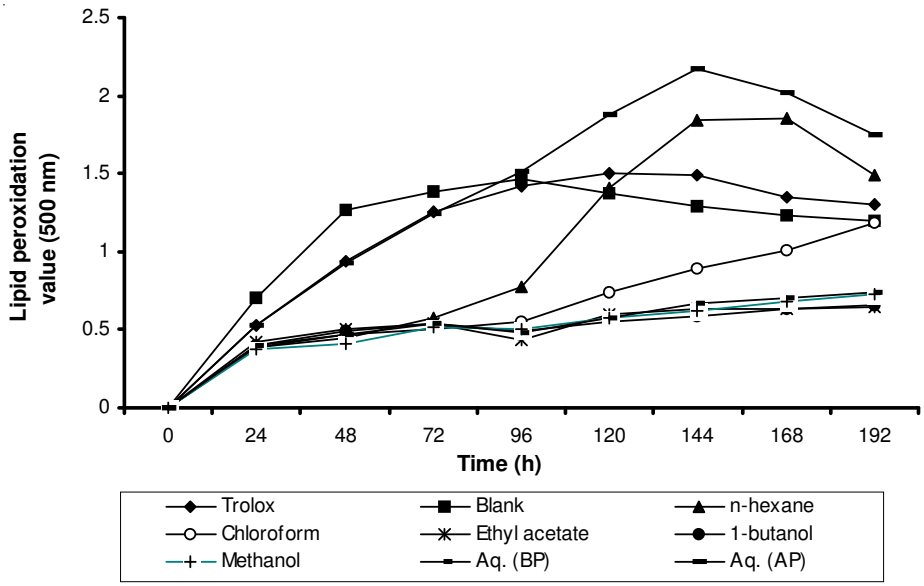
Fig. 3. % DPPH remaining values of the extract of (A) leaves (chloroform and ethyl acetate five times conc.) (B) stem (*n*-hexane and ethyl acetate two times conc., methanol three times conc., chloroform, 1-butanol, aq. AP and aq. BP five times conc.) (C) fruit (1-butanol, ethyl acetate, methanol and *n*-hexane two times conc. chloroform three times conc.) of *M. nigra* as a function of time

proteins leading to cell inactivation[44]. Many disorders like hyperglycaemia have been ascribed to development of oxidative stress due to increased lipid peroxide production[45]. Antioxidants are able to inhibit deterioaration of bone and cartilage resulting from lipid peroxidation and suppress the inflammation[30]. Lipid peroxidation values of extracts from different parts of *M. nigra* were determined using linoleic acid peroxidation system. Peroxyl radicals formed due to oxidation of linoleic acid are allowed to oxidize ferrous to ferric form, which subsequently reacts with

thiocyanate to form a coloured complex. In the presence of samples, oxidation of linoleic acid is halted proportionate to the amount of antioxidant components present in the sample. Fig. 4 demonstrated that most of the fractions of the *M. nigra* were possessing higher lipid peroxidation values than that of trolox. Ethyl acetate, aqueous (BP) and 1-butanol fractions of all the three parts possessed appreciable lipid peroxidation inhibition activity.



(A)



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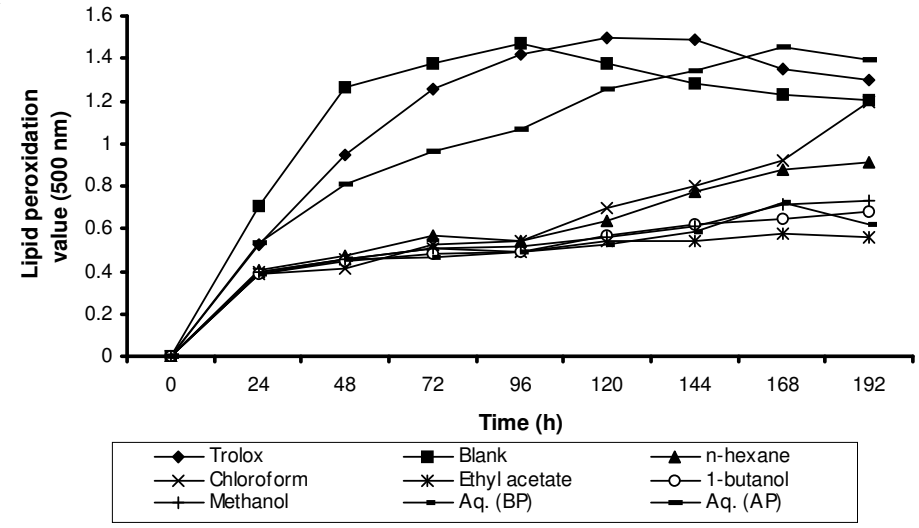

(C)

Fig. 4. Peroxidation values of extracts of (A) leaves (B) stem and (C) fruit of *M. nigra* using thiocyanate method

Conclusion

The data presented here shows that *M. nigra* extracts have great antioxidant potential and radical scavenging activity and may be used an alternative to the synthetic antioxidants. In traditional medicinal system of Indo-Pak sub-continent *in vivo* efficacy of *M. nigra* extracts against diabetes mellitus or other degenerative diseases may be attributed to radical scavenging and antioxidant activity of the plant.

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