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Protective effects of *Koelreuteria paniculata* Laxm. on oxidative stress and hydrogen peroxide-induced DNA damage

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Abstract

In this study, n-butanol fraction (KBF) and aqueous fraction (KAF) isolated from methanol extract of *Koelreuteria paniculata* Laxm. leaves were evaluated for their antioxidant potential using (ABTS) 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-2-picrylhydrazyl) assay, Reducing power assay and Superoxide anion radical scavenging assay. These fractions were also studied for their DNA protective activity based on hydrogen peroxide-induced DNA damage using calf thymus DNA. Both fractions exhibited good scavenging potential in ABTS assay, DPPH assay, reducing power assay and superoxide anion radical scavenging assay. The KBF and KAF showed percent antioxidant activity of 73.24 and 81.18 in DPPH assay, 90.93 and 60.27 in ABTS assay, 47.30 and 73.70 in reducing power assay and 77.5 and 60.22 in superoxide anion radical scavenging assay at highest tested concentration respectively. Both fractions also showed protection to calf thymus DNA against hydroxyl radical damage generated as result of Fenton's chemistry in DNA protection assay.

Key words: *Koelreuteria paniculata*; Antioxidant potential; Fenton's chemistry; DNA damage; DPPH assay; ABTS assay

Introduction

Normally endogenous and exogenous antioxidant defense system plays an important role in ameliorating the oxidative stress in the body but sometimes there is excessive production of free radicals which cause damage to different biomolecules (Halliwell and Gutteridge, 1989). Normally, ROS are responsible to help several signal transduction and intercellular communications (Valko et al., 2004). Overproduction of these reactive oxygen species cause number of pathological effects such as causing lipid peroxidation, protein peroxidation, DNA damage and cellular degeneration leading to cardiovascular disease,

ageing, cancer, inflammatory diseases and a variety of other disorders (Finkel and Holbrrok, 2000; Knight, 1995). To keep balance between the production of free radicals and defense system of the body, there is need of antioxidants in our diet (Yu et al., 2002). Synthetic antioxidant compounds such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, tertiary butylhydroquinone etc. are often added to various foodstuffs to prevent degradation resulting from oxidation. But the use of these synthetic antioxidants in food items has shown negative effects on health. Further, the use of synthetic antioxidants in foods is under strict regulation, because of uncertain nature in relation to their safety (Iqbal et al., 2007; Jeong et al., 2004). Hence, much interest has been taken in searching plant-derived antioxidants which provide health benefits.

Numerous antioxidants from plants have been verified to be useful for prevention/attenuation of disease progression (Zhang et al., 2006). The protective role played by plant products is because of several constituents they harbour such as enzymes, phenols, flavonoids, vitamins etc. The antioxidants are helpful in delaying and preventing oxidation catalyzed by ROS (Velioglu et al., 1998; Head, 1998; Edge et al., 1997). Polyphenols are the class of aromatic compounds occurring in various vegetables and fruits which form a major portion of our diet. These compounds have antioxidant properties, which help in protecting our body from oxidative damage (Bennick, 2002).

Antioxidant compounds derived from plant sources such as vitamin E, vitamin C, phenolic acids, flavonoids, catechins, procyanidins, and anthocyanins are recommended as nutraceuticals/functional foods (Ferguson, 2001; Arouma, 1994). Phenolic compounds have been known to possess antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory activities (Breinholt, 1993; Duthie et al., 2000; Shahid et al., 1995). Natural pure compounds are reported to have pronounced chemopreventive effect through modulation of molecular events that damage DNA and other biomolecules (Tahara et al., 2005). Antioxidant phytochemicals are necessary in food due to their potential to reduce free radical-mediated damage in cell and tissues in the body (Jin et al., 2004; Wongkham et al., 2001). *Koelreuteria paniculata* Laxm. (Family Sapindaceae) known as golden rain tree is a drought resistant tree grown for its abundant summer flowers and its papery lantern like fruits. Flowers are used as source of yellow dye and in traditional medicines. Keeping in mind, the medicinal role of plant in traditional medicine, it is planned to study the antioxidant/genoprotective activity of n-butanol and aqueous fraction isolated from leaves of *Koelreuteria paniculata* Laxm. for their eventual application as cancer chemopreventive agents**.**

Materials and methods

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric chloride, Nicotinamide adenine dinucleotide (NADH), phenazine methosulphate, nitrobluetetrazolium, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), BHT and L-Ascorbic acid were obtained from HiMedia Pvt. Limited. Mumbai, India. Ascorbic acid and Rutin were obtained from Sigma (St. Louis, MO, USA). Calf thymus DNA was purchased from Genei Pvt. Ltd., Banglore (India). All other reagents were of analytical grade (AR).

Plant material

Collection of plant material

Leaves of the plant were collected in month of November from Botanical garden of Guru Nanak Dev University, Amritsar, Punjab (India). The specimen was identified by the Herbarium of Department of Botanical and Environmental Sciences and Voucher specimen No. 0409/HRB was deposited in herbarium of the same Department.

Extraction and Isolation

The leaves were washed with running tap water and dried in shade for week. The dried leaves were powdered and extracted three times with 80 % methanol (3200 ml) and extract was concentrated using rotary evaporator. The methanol mother extract (35 g) was then fractionated using separating funnel with different organic solvents viz. Hexane (900 ml), chloroform (900 ml), ethyl acetate (900 ml), n-butanol (900 ml) in the order of increaseng polarity to obtain KCF (Chloroform fraction; 5.12 g), KEA (Ethyl acetate fraction; 4.56 g), KBF (n-Butanol fraction; 10.23 g) and KAF (Aqueous fraction; 8.45 g) (Figure 1). Finally all the fractions were concentrated using rotary vacuum evaporator (Buchi Rotavapor R-210). In the present study, KBF and KAF fractions were tested for their antioxidant/genoprotective activity.

Phytochemical analysis

UV spectroscopy

UV spectrum of the fractions was recorded in DMSO using Shimadzu UV-VIS 160-A Spectro-photometer.

Determination of total phenolic content (TPC)

Total phenolic content (TPC) of the test samples was determined using Folin-Ciocalteu method (Yu et al., 2002) employing gallic acid as standard. To 100 µl of test sample (100 µg/ml) was added 900 µl of double distilled water. To this 500 µl of Folin-Ciocalteu reagent was added. This was followed by the addition of 1.5 ml of 20 % sodium carbonate. The volume was raised to 5 ml. The mixture was then incubated for 2 h at room temperature. Finally absorbance was taken at 765 nm (Systronics 2202 UV–Vis Spectrophotometer). Phenolic content was calculated as gallic acid equivalents (GAE) on the basis of standard curve of gallic acid.

Determination of total flavonoid content (TFC)

The method given by Kim et al. (2003) was used for determination of total flavonoid content (TFC) employing rutin as a standard. Total flavonoid content of the test sample was determined using colorimetric method. To 1 ml of 100 µg/ml test fraction, 4 ml of double distilled water was added followed by addition of 300 µl of $NaNO_2$ and 300 µl of $AlCl_3$, which was incubated for 5 minutes. To this 2 ml of NaOH was added and final volum-

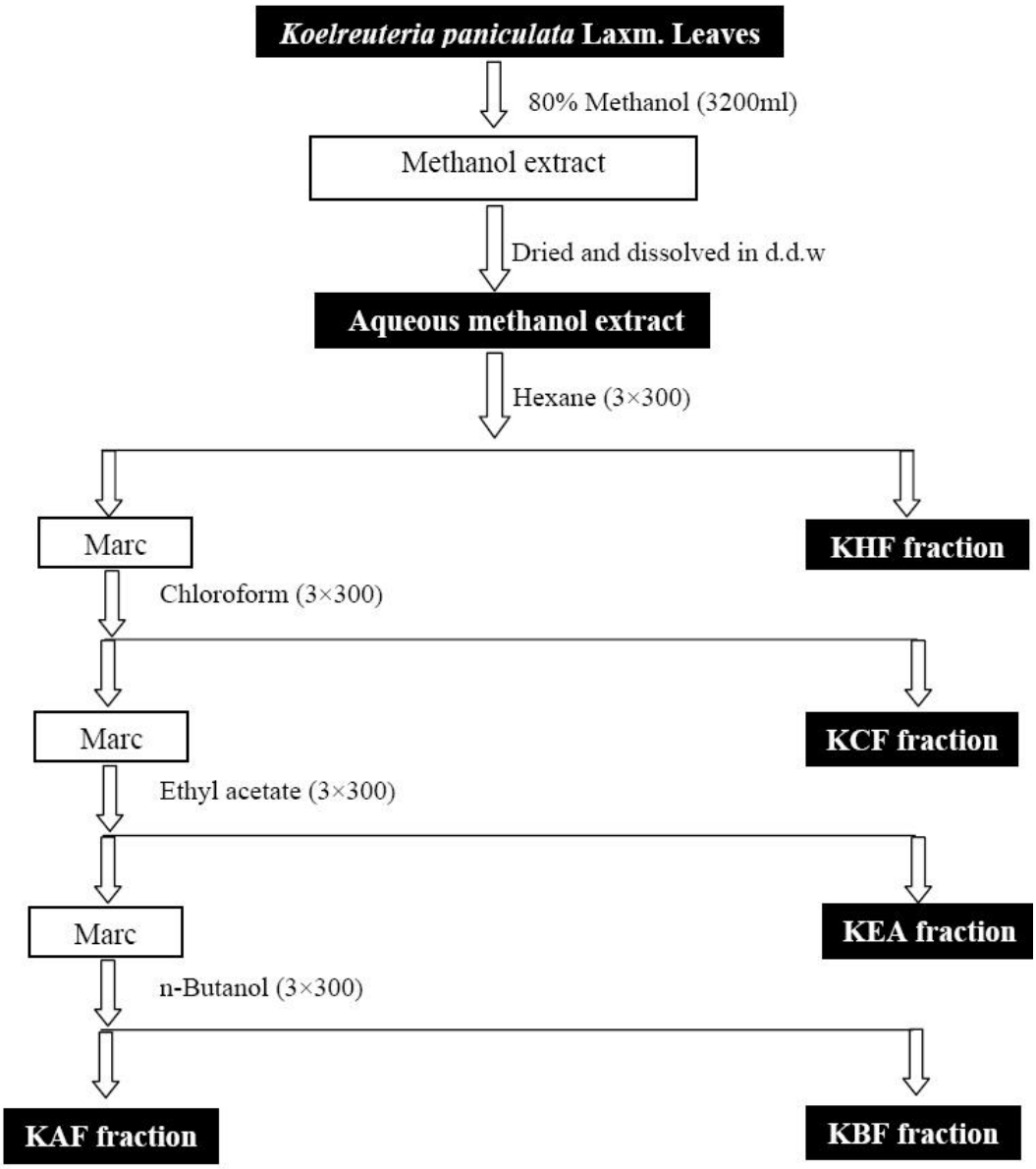

Figure 1. Flow chart representing extraction procedure for the preparation of KBF and KAF fractions of *K. paniculata* Laxm.

e was raised to 10 ml. Finally absorbance was taken at 510 nm (Systronics 2202 UV–Vis Spectrophotometer). Total flavonoid content was then expressed as rutin equivalents (RE) in mg/g of dry sample.

DNA Protection assay

DNA protection assay was performed using Calf thymus DNA with slight modifications (Lee et al., 2002). Calf thymus DNA (5 µg) was incubated with Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) containing test sample and the final volume of the mixture was raised up to 20 µl. The mixture was then incubated for

30 min at 37°C followed by addition of loading dye and electrophoresis was carried out in TAE buffer (40 mM Tris base, 16 mM acetic acid 1mM EDTA, pH 8.0) at 50 V for 2 h then DNA was stained by ethidium bromide. Finally gel was analyzed using Gel Doc XR system (Bio-Rad, USA). Rutin was used as standard.

Antioxidant Assays

$ABTS^{\cdot+}$ radical scavenging assay

$ABTS^{\cdot+}$ scavenging assay was carried out by the method given by Re *et al.* (1999). ABTS cation was generated by reacting 7 mM ABTS stock solution and 2.45 mM Potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS cation solution was diluted with ethanol (2 ml) to an absorbance of 0.700 (±0.02) at 734 nm (Systronics 2202 UV–Vis Spectrophotometer). 100 µl of test solution was added to the diluted ABTS cation solution and absorbance reading was taken up to 5 min. BHT was used as standard. Radical scavenging activity % = $(Abs_0 - Abs_1/Abs_0) \times 100$. Where Abs_0 is the absorbance of ABTS solution and Abs_1 is the absorbance of reaction mixture (containing test sample & ABTS solution).

DPPH-radical scavenging assay

DPPH scavenging activity was carried out by the method of Blois (1958) with slight Modifications. Different concentrations (40-200µg/ml) of test samples (100 µl) of *Koelreuteria paniculata* were dissolved in methanol and taken in test tubes in triplicates. Then 2 ml of 0.1 mM methanol solution of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was added to each of the test tubes and were shaken vigorously and incubated in dark for 30 minutes. After 30 minutes, absorbance was taken at 517 nm (Systronics 2202 UV–Vis Spectrophotometer). The control was prepared without any test samples. Ascorbic acid was used as standard. Radical scavenging activity % = $(Abs_0 - Abs_1/Abs_0) \times 100$. Where Abs_0 is the absorbance of DPPH solution and Abs_1 is the absorbance of reaction mixture (containing test sample & DPPH solution).

Reducing power assay

Reducing potential of the test samples was determined using the method of Oyaizu (1986). Different concentrations (40-200 µg/ml) of test samples (1 ml) of *Koelreuteria paniculata* were dissolved in methanol and taken in test tubes in triplicates. To the test tubes, 2.5 ml of phosphate buffer (pH 6.6, 0.2 M) and 2.5 ml of 1% Potassium ferricyanide solution was added. These contents were mixed well and were incubated at 50˚C for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid (TCA) was added and kept for centrifugation at 3000 rpm for 10 minutes. After centrifugation, 2.5 ml of supernatant was taken and 2.5 ml of double distilled water was added, followed by addition of 0.5 ml of 0.1% ferric chloride. The absorbance was measured spectrophotometrically at 700 nm (Systronics 2202 UV–Vis Spectrophotometer). Increase in absorbance of reaction mixture was interpreted as increase in reducing ability of the test samples and the results were compared with ascorbic acid which was used as reference compound. The percentage of reduction of the sample as compared to the standard (ascorbic acid) was calculated using the formula:

% Reducing power = [1-(1-As/Ac) ×100]. Where, Ac = absorbance of standard compound at maximum concentration tested and As = absorbance of sample.

Superoxide anion radical scavenging assay

The measurement of superoxide anion scavenging activity of test samples of *K. paniculata* was performed based on the method described by Nishikimi et al. (1972) with slight modifications. About 1 ml of nitroblue tetrazolium (NBT) solution (156 µM prepared in 100 mM phosphate buffer, pH 7.4), 1 ml of NADH solution (468 µM prepared in 100 mM phosphate buffer pH 7.4) and test samples concentrations (100-500 µg/ml) were mixed and the reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM) prepared in phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated at 25˚C for 5 min and the absorbance at 560 nm (Systronics 2202 UV–Vis Spectrophotometer) was measured against the control samples. Rutin was used as reference compound. Antioxidant activity % = (Abs_0 - Abs_1/Abs_0) × 100. Where, Abs_0 is the absorbance of control (reaction mixture without test sample) and Abs_1 is the absorbance of test sample.

Statistical Analysis

Results were expressed as mean ± standard error. Inhibitory concentration (IC_{50}) value was calculated by regression analysis. One-way analysis of variance (ANOVA) and High range statistical domain (HSD) using Tukey's test were carried out to determine significant differences among means ($p \leq 0.05$).

Results and Discussion

Phytochemical analysis

Polyphenols obtained from plants are the group of secondary metabolites which act as primary antioxidants or free radical terminators. So, it is necessary to determine the total amount of phenols in the plant under study. Polyphenol compounds occur in both edible and inedible plants and have been known for their antioxidant activity. The antioxidant activity of phenols is chiefly because of their redox properties which could play a vital function in diminution of free radicals. In this study, total phenolic content (TPC) was found to be 210 and 240 mg/g GAE in KBF and KAF fractions respectively and Total flavonoid content (TFC) was found to be 150 and 180 mg RE/g in KBF and KAF fractions respectively (Table 1). The UV spectrum of KBF and KAF fraction gives λ_{max} at 268 and 265 nm which shows the presence of polyphenols in these fractions.

Table 1. Flavonoid and phenol contents in the KBF and KAF fractions.

Extract	TPC (mg GAE/g dry wt. of extract)	TFC (mg RE/g dry wt. of extract)
KBF fraction	210	150
KAF fraction	240	180

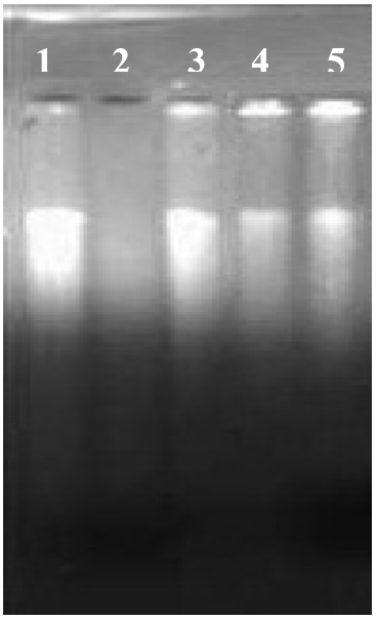

Figure 2. Effect of *Koelreuteria paniculata* leaf fractions on the protection of calf thymus DNA against hydroxyl radical generated by the H_2O_2. Lane 1: Calf thymus DNA (control), Lane 2: Calf thymus DNA + Fenton's reagent (for DNA damage), Lane 3: Calf thymus DNA + Fenton's reagent + Rutin (standard), Lane 4: Calf thymus DNA + Fenton's reagent + KBF fraction, Lane 5: Calf thymus DNA + Fenton's reagent + KAF fraction

DNA protection assay

It is well known that hydroxyl radicals are generated under aerobic conditions by Fenton's reagent. These radicals cause oxidative damage which includes single and double strand breaks in DNA, base modification and cross linking of DNA with proteins. So tissue uncovered to hydroxyl radicals may lead to cancer, cardiovascular diseases, cataract and neurological disorders (Cadet et al., 1997; Vaya et al., 2001). These hydroxyl radicals attack biomolecules such as proteins, nucleic acids and polysaccharides (Pellegrini et al., 1999). Kumar et al. (2011) studied the chloroform and ethyl acetate fractions obtained from *Koelreuteria paniculata* methanol extract for pUC18 DNA protection studies and reported that both fractions were capable of protecting DNA. In calf thymus DNA protection studies, the exposure of native DNA to Fenton's reaction caused fragmentation of DNA with disappearance of DNA bands. It is clear from the results that the addition of KBF and KAF fractions to reaction mixture, protects DNA (Figure 1; lane 4 &5) at the concentration of 250 µg/ml probably by scavenging of the ·OH radicals generated by Fenton's reaction because in the absence of extracts, there is complete fragmentation of DNA (Figure 2; lane 2). Rajkumar et al. (2010) evaluated *Berginia ciliata* rhizome extracts for scavenging of hydroxyl radicals in plasmid nicking assay using pBR322 plasmid DNA.

Antioxidant assays

$ABTS^{+\bullet}$ assay is most commonly used method for determination of antioxidant activity. The abstraction of H-atoms by stable $ABTS^{+\bullet}$ radical leads to decrease of absorbance of the reaction mixture which can be spectrophotometrically measured at 734 nm

Table 2. IC50 values for KBF and KAF fractions in different *in vitro* free radical scavenging assays.

S .No.	Assay	KBF fraction IC_{50} value (µg/ml)	KAF fraction IC_{50} value (µg/ml)
1	$ABTS^{\cdot+}$ assay	72.65	152.18
2	DPPH assay	135.07	111.04
3	Reducing power assay	N.D	132.79
4	Superoxide anion radical scavenging assay	145.56	332.60

ND: Not detemined

(Re et al., 1999). ABTS radical cation scavenging method is mostly applied for the determination of radical scavenging potential of pure compounds, beverages and aqueous mixtures (Pellegrini et al., 1999). Kumar et al. (2011) reported that chloroform and ethyl acetate fractions of *K. paniculata* exhibited good antioxidant potential using different *in vitro* assays. In this study, the KBF fraction possessed scavenging activity of 90.93% (IC_{50} 72.65 µg/ml) while KAF fraction exhibited 60.27% (IC_{50} 152.18 µg/ml) inhibition at the concentration of 200 µg/ml (Table 2). The standard compound BHT showed 51.6% inhibition at same concentration (IC_{50} of 197.55 µg/ml) (Figure 3). Phytochemical analysis showed good amount of phenolic and flavonoid content in both the fractions so this ABTS cation radical scavenging potential may be a contribution of these phytoconstituents. Ozsoy et al. (2009) studied the extracts of *Amaranthus lividus* for ABTS cation radical scavenging and reported that activity of extracts is comparable to standard antioxidants such as gallic acid and BHA.

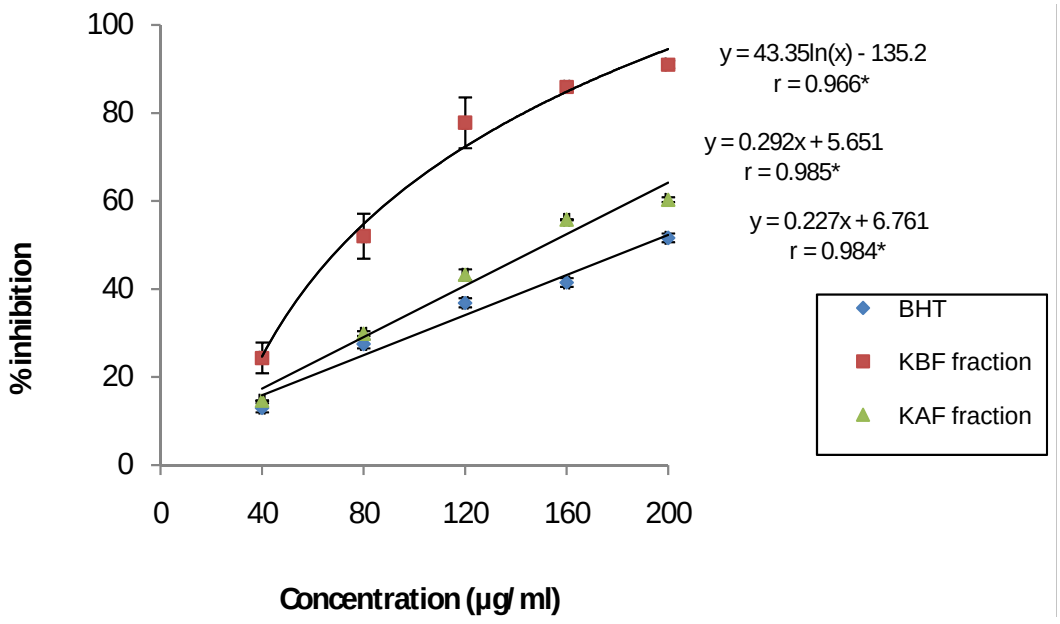

Figure 3. Scavenging effect of different concentrations of KBF and KAF fractions of *K. paniculata* leaves and BHT (standard) on ABTS radicals. (*p≤0.05)

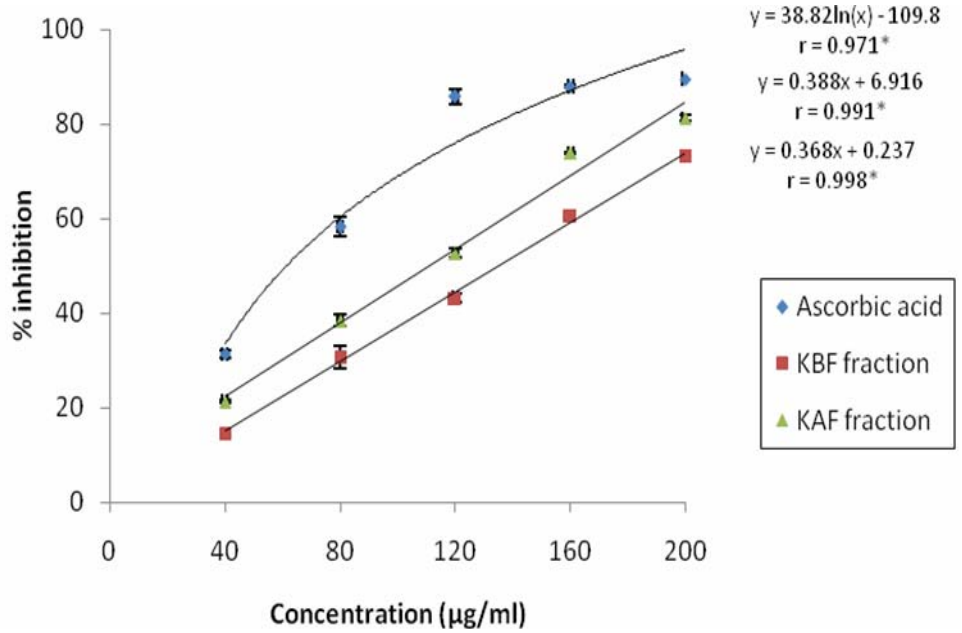

Figure 4. Inhibitory effect of different concentrations of KBF and KAF fractions of *K. paniculata* leaves and Ascorbic acid (standard) on DPPH radicals. (*p≤0.05)

Antioxidants compounds have been screened for the free radical scavenging activity by widely used method known as DPPH assay (Molyneux, 2004). DPPH gives rise to deep violet color in methanol characterized by an absorption band at about 517 nm. On the addition of antioxidant substance to DPPH solution, antioxidant substance donates electron

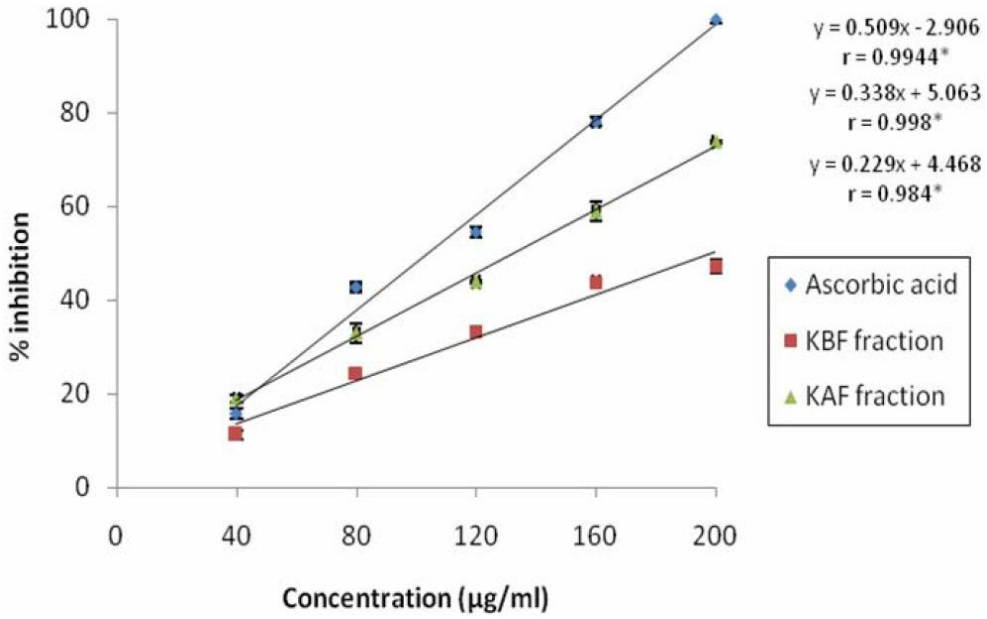

Figure 5. Reducing potential of KBF extract and KAF fractions *K. paniculata* leaves in comparison to Ascorbic acid (standard). (*p≤0.05)

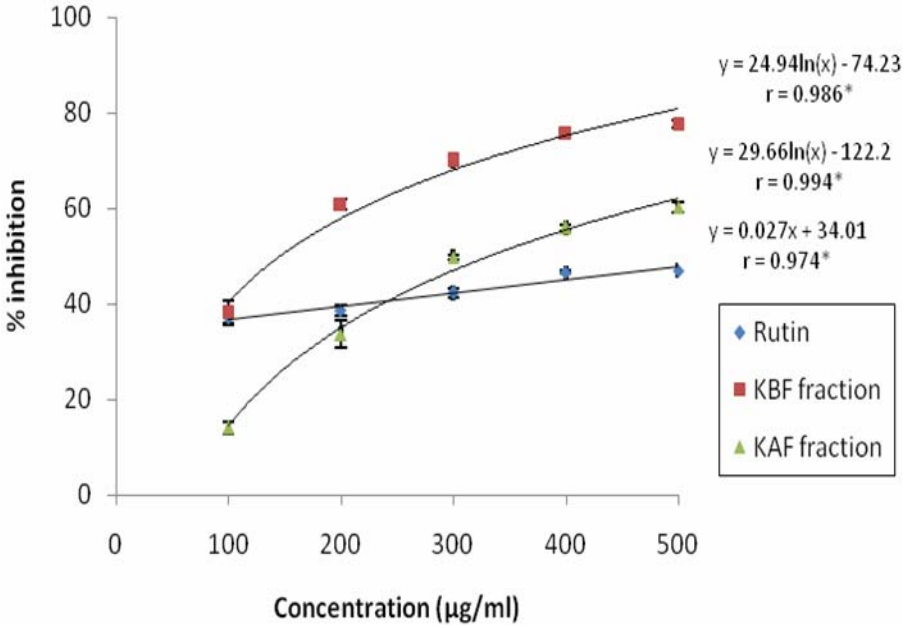

Figure 6. Scavenging effect of different concentrations of KBF and KAF fractions of *K. paniculata* leaves and Rutin (standard) on superoxide anion radicals. (*p≤0.05)

or H- atom resulting in loss of violet color and appearance of yellow color. So, on the addition of antioxidant substance to DPPH solution, its absorbance starts decreasing which can be measured spectrophotometrically. In this study, the KBF and KAF fraction showed 73.24% (IC_{50} of 135.07 µg/ml) and 81.18% (IC_{50} 111.04 µg/ml) inhibition of DPPH radicals at the concentration of 200 µg/ml. Figure 4 shows the dose response curve of both the fractions obtained from *K. paniculata*. The kinetic studies were carried out to determine the scavenging ability of fractions as a function of time, revealed that at early stages of reaction, the fractions showed lesser tendency to quench DPPH radicals, which increased with time. The standard compound ascorbic acid showed 89.53% inhibition at concentration of 200 µg/ml (IC_{50} of 55.88 µg/ml). The scavenging activity of KAF fraction was more than KBF fraction, since KAF fraction showed more phenolic and flavonoid content than KBF fraction. Hence, DPPH scavenging activities were in concordance with phenolic and flavonoid content of fractions (Table 1).

The reducing ability generally depends on the presence of reductones, which possess antioxidative activity by breaking the free radical chain and donating a hydrogen atom. The reductants (antioxidants) in plant extracts cause the transformation of Fe^{3+}/ferricyanide complex to the ferrous form. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue color at 700nm. (Duh, 1998; Yen and Duh, 1994). It has been found that the reducing capacity of both the fractions increased in dose-dependent manner. At the concentration of 200 µg/ml, the KBF and KAF fraction exhibited 47.30% (IC_{50} N.D) and 73.70 % (IC_{50} 132.79 µg/ml) inhibition as compared to standard ascorbic acid (Figure 4). These results were in concordance with their phenolic and flavonoid content. More is TPC/TFC content, high is reducing potential of plant extracts.

It has been reported that antioxidant properties of some flavonoids are due to scavenging of superoxide anion radicals. Superoxide anions cause damage to lipids, proteins

and DNA (Pietta, 2000; Cos et al., 1998). In this study, two fractions of *K. paniculata* were tested for their potential to scavenge superoxide anions using method in which $O_2^{\bullet-}$ are generated by non-enzymatic reaction of phenazine methosulfate in presence of NADH and molecular oxygen (Nishikimi et al., 1972). The percent inhibition of superoxide anion radical by KBF extract and KAF fraction was found to be 77.75 (IC_{50} 145.56 µg/ml) and 60.22 (IC_{50} 332.60 µg/ml) at the concentration of 500 µg/ml. The standard compound rutin showed 47.01% inhibition of superoxide anions at the same concentration (Figure 5). Antioxidants are capable of inhibiting the blue NBT formation (Parejo et al., 2002; Khalil et al., 2008). The decrease of absorbance at 560 nm with antioxidant compounds indicates consumption of superoxide anion in a reaction mixture.

According to the data obtained from the present study, both fractions from *K. paniculata* were found to be effective in different *in vitro* antioxidant assays and DNA protection assay. These can be used for retarding lipid oxidation in food stuffs, minimizing production of oxidation reaction toxic products and finally for prolonging and maintaining nutritional quality of foods and medicines. Thus, these sources can be used as new sources of natural antioxidants for food and as nutraceuticals.

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