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Studies on the Antioxidant/Genoprotective Activity of Extracts of *Koelreuteria paniculata* Laxm

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Abstract

Oxidative injury arising from reactive oxygen species (ROS) appears as the primary mechanism underlying many of human diseases such as cancer, neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders. Plant extracts or secondary metabolites have served as antioxidants in phytotherapeutics medicines to protect against various diseases for centuries. *Koelreuteria paniculat*a Laxm. is Chinese traditional medicinal plant used in eye related diseases. The 80% methanol mother extract (KPE) along with its hexane fraction (KPF) from the leaves of *Koelreuteria paniculat*a Laxm. were tested for antioxidant and genoprotective potential. The antioxidant potential was checked using ABTS, DPPH (2,2-diphenyl-2-picryl-hydrazyl), Reducing power and Superoxide anion radical scavenging assay and genoprotective activity against the DNA damage induced by Fenton's reagent using DNA protection studies. The analysis of free radical scavenging activities of the KPE and KPF revealed a concentration-dependent antiradical activity in all the assays. The KPE exhibited IC_{50} of 54.54 µg/ml in ABTS, 115 µg/ml in DPPH, 110 µg/ml in reducing power and 135 µg/ml in superoxide anion radical scavenging assay while that KPF was found to be very poor in radical scavenging in all the above assays. The phytochemical analysis showed good amount of phenolic and flavonoid compounds in KPE while the KPF fraction lacks phenolic compounds. The KPE extract and KPF fraction both showed DNA protective effect in Calf thymus/pUC18 DNA protection studies. The activity of KPE may be attributed to its polyphenolic constituents which needs further investigation.

Keywords: Oxidative injury; DNA damage; Fenton's reagent; *Koelreuteria paniculata* Laxm.

1. Introduction

Reactive oxygen species (ROS) are produced in the human body during a variety of metabolic processes or penetrate in the body from environment. ROS cause cellular damage by reacting with the various biomolecules of body such as membrane lipids, nucleic acid, proteins and enzymes. ROS have been involved in pathogenesis of number of ailments including cancer, aging, arthritis, cardiovascular diseases, inflammation, diabetes mellitus, renal failure and brain dysfunction [19]. Antioxidants can act as scavengers of these free radicals. Use of antioxidant compounds in food stuffs may prevent free radical-induced lipid oxidation of food [1]. Plants posssess many phytochemicals with various bioactivities including antioxidant, anti-inflammatory and anticancer [36]. In recent years, great attention has been paid to antioxidant properties of plants that may be used for human consumption. The comprehensive estimation of antioxidant activity of natural plant products using different test methods has been shown to be vital in identifying both the antioxidant and pro-oxidant activities of these compounds [26]. Plants contain substantial amounts of phytochemical antioxidants as phenolics, carotenoids, flavonoids, tannins which can be used to scavenge the excess free radicals present in the body. The antioxidant potential of phenolics is because of their redox properties which allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators [4]. Since the past few years, Genetic Toxicology laboratory of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar has been seriously involved in bioprospecting the medicinal plants for the presence of antimutagenic/antigenotoxic phytochemicals [9-17]. *Koelreuteria paniculata* Laxm. (Family Sapindaceae) known as golden rain tree is a drought resistant tree grown for its abundant summer flowers and its papery lanterns like fruits. Flowers are used as source of yellow dye and in traditional medicines. The present work was designed to investigate the antioxidant/genoprotective potential of *Koelreuteria paniculata* Laxm. extract (KPE) and *Koelreuteria paniculata* Laxm. fraction (KPF) of leaves of *Koelreuteria paniculata* Laxm.

2. Materials and methods

2.1. 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. Gallic acid, Ascorbic acid, Rutin and BHT were obtained from Sigma (St. Louis, MO, USA). Plasmid pUC18 and Calf thymus DNA were purchased from Genei Pvt. Ltd., Banglore. All other reagents were of analytical grade (AR).

2.2. Plant material

2.2.1. Collection of plant material

The leaves of the plant *Koelreuteria paniculata* were collected from Botanical garden of Guru Nanak Dev University, Amritsar, Punjab. The specimen was identified by Mr. Ramprasad Herbarium Incharge in the same department and voucher specimen No. 0409/HRB was deposited in herbarium of the Department.

2.2.2. Extraction and Isolation

Leaves were washed with running tap water to remove dust impurities and finally air dried, protected from direct sunlight. The dried leaves were ground to fine powder and extracted three times with 80% methanol and concentrated using rotary vacuum evaporator (Buchi Rotavapor R-210) to obtain KPE. KPE was then made aqueous with distilled water and fractionated with non polar solvent n-hexane. The n-hexane fraction was concentrated using rotary vacuum evaporator to obtain KPF (Flow chart 1).

2.3. Antioxidant assays

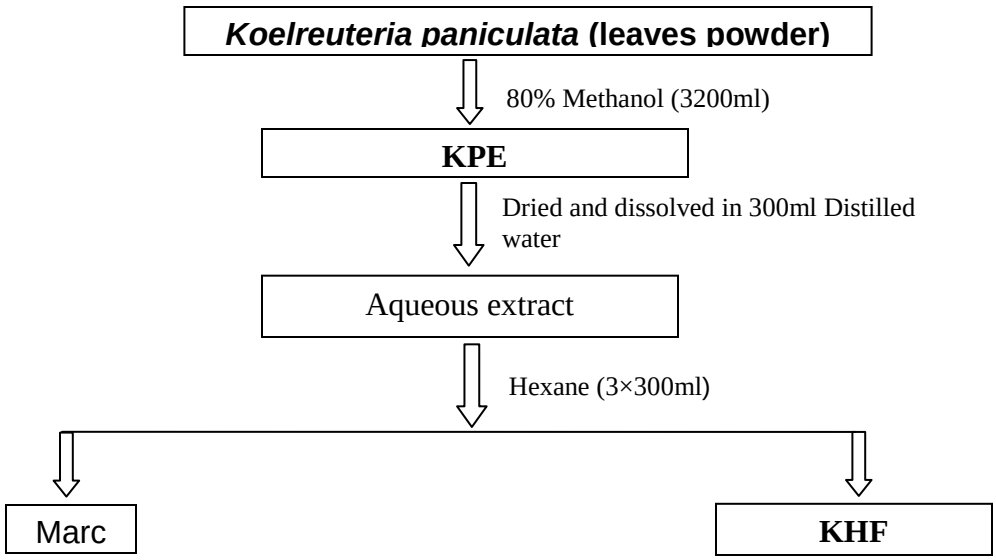
2.3.1 $ABTS^{\cdot+}$ radical scavenging assay

$ABTS^{\cdot+}$ scavenging assay was carried out by the method given by Re *et al.* (1999) [29]. ABTS cation was generated by reacting 7mM ABTS

stock solution and 2.45mM 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 to an absorbance of 0.70 (±0.02) at 734 nm. 100 µl of test solution was added to the diluted ABTS cation solution and absorbance reading was taken up to 5 min (Systronics 2202 UV–Vis Spectrophotometer, India). BHT was used as antioxidant standard.

Radical scavenging activity % = A_0 - $A_1/A_0 \times 100$

where A_0 is the absorbance of ABTS solution. A_1 is the absorbance of reaction mixture (containing test sample & ABTS solution).



Flow chart 1: Isolation of KPE/KPF of *K. paniculata*

2.3.2 DPPH-radical scavenging assay

DPPH scavenging activity was carried out by the method of Blois (1958) [3] with slight modifications. Different concentrations (40-200 µg/ml) of test samples of *Koelreuteria paniculata* were dissolved in methanol and taken in test tubes in triplicates. Then 2ml 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. After 30 minutes, absorbance was taken at 517 nm using UV-VIS spectrophotometer (Systronics 2202 UV–Vis Spectrophotometer, India). The control was prepared without any test samples. Ascorbic acid was used as antioxidant standard.

Radical scavenging activity % = A_0 - $A_1/A_0 \times 100$

where A_0 is the absorbance of DPPH solution. A_1 is the absorbance of reaction mixture (containing test sample & DPPH solution).

2.3.3. Reducing power assay

Reducing potential of extract/fraction was determined using the method of Oyaizu (1986) [27]. Different concentrations (40-200 µg/ml) of test samples 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, 0.2M) 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, 2.5ml of 10% trichloroacetic acid (TCA) was added and kept for centrifugation at 3000 rpm for 10 minutes. After centrifugation, 2.5ml of supernatant was taken and to this, 2.5ml of double distilled water was added, followed by addition of 0.5ml of 0.1% ferric chloride. The absorbance was measured spectrophotometrically at 700nm using UV-VIS spectrophotometer (Systronics 2202 UV–Vis Spectrophotometer, India). 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.

2.3.5. 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) [25] 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 was measured against the control samples using UV-VIS spectrophotometer (Systronics 2202 UV–Vis Spectrophotometer, India). Rutin was used as the reference compound. All the tests were performed in triplicate and the results averaged.

Antioxidant activity % = $A_0 - A_1/A_0 \times 100$

where A_0 is the absorbance of control (reaction mixture without test sample). A_1 is the absorbance of test sample

2.4. DNA Protection assay

A DNA protection assay was performed using supercoiled pUC18 plasmid DNA/calf thymus DNA [21] with slight modifications. Plasmid DNA/ calf thymus DNA (5µg) was incubated with Fenton's reagents (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) containing test sample (50 µg/ml and 250 µg/ml in pUC18 DNA and Calf thymus DNA protection studies respectively) and the finally 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 (40mM Tris base, 16mM acetic acid 1mM EDTA, pH 8.0) at 60 V for 2.5 h. DNA was analyzed followed by ethidium bromide staining using Gel Doc XR system (Bio-Rad, USA). The gallic acid and rutin was used as standard.

2.5. Phytochemical analysis

2.5.1. Determination of total phenolic content

The total phenolic content (TPC) of the KPE/KPF was determined using Folin-Ciocalteu method of Yu *et al*. (2002) [39] employing gallic acid as standard. To100 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 of mixture was made up to 10 ml with distilled water and allowed to stand for 2 h. Finally absorbance was taken at 765 nm. The phenolic content was calculated as gallic acid equivalents (GAE) on the basis of standard curve of gallic acid.

2.5.2. Determination of total flavonoid content

The method given by Kim *et al*. (2003) [18] 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 1ml of 100µg/ml extract /fractions, 4ml 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 mixture 2ml of NaOH was added and the final volume was raised to 10ml. Finally absorbance was taken at 765 nm. The flavonoid content was calculated as rutin (mg/g) equivalents. The total flavonoid content was then expressed as rutin equivalents (RE) in mg/g of dry sample.

3. Statistical analysis

The experimental results were expressed as mean ± standard error (SE) of three parallel measurements. 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.

4. Results

4.1. ABTS$^+$ radical scavenging assay

The KPE exhibited radical scavenging activity of 98.35% (IC_{50} of 54.54 µg/ml) while KPF fraction showed 29.11% of inhibition at the concentration of 200 µg/ml. The standard compound BHT showed 51.6% inhibition at concentration of 200 µg/ml (IC_{50} of 197.55 µg/ml) (Fig. 1).

4.2. DPPH Assay

The KPE extract showed promising free radical scavenging effect with inhibition 84.93% (IC_{50} of 115 µg/ml) while KPF showed poor inhibition of DPPH radicals with inhibition of 19.86% concentration of 200 µg/ml (Fig. 2). The antioxidant standard compound ascorbic acid showed 89.53% inhibition concentration of 200 µg/ml (IC_{50} of 55.88µg/ml).

4.3. Reducing Power Assay

The reducing capacity of a compound Fe^{3+}/ferricyanide complex to ferrous form may serve as indicator of its antioxidant capacity [38]. At the concentration of 200 µg/ml, the KPE and KPF exhibited 85.63% and 15.00 % of inhibition as compared to standard ascorbic acid (Fig. 3).

4.4 Superoxide anion radical scavenging assay

The superoxide radical activity of extract/fraction was measured by non-enzymatic PMS-NADH system. The percent inhibition of superoxide anion radical generation by KPE and KPF was found to be 79.64 and 7.60 % at the concentration of 500 µg/ml. The standard compound rutin showed 47.01% inhibition of superoxide anions at the same concentration (Fig. 4).

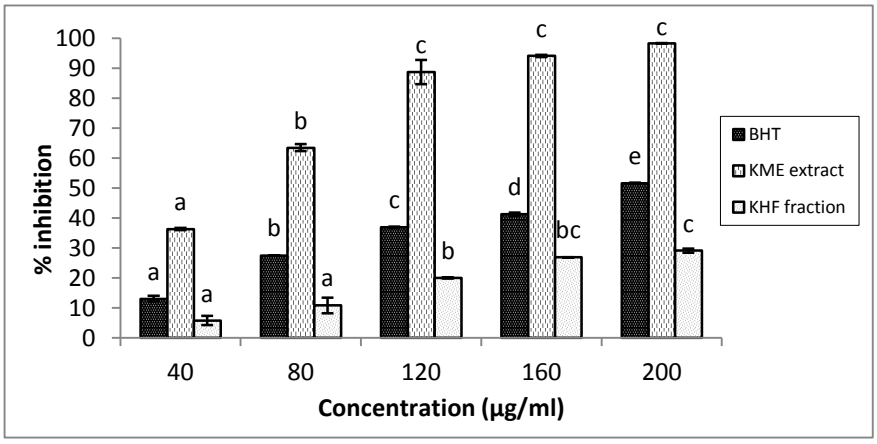


Figure 1. Scavenging effects of KPE and KPF of leaves and standard BHT on ABTS radicals. Different letters (a,b,c,d &e) with in the same extract/fraction indicates that these concentrations are significantly different from each other at the level of $p \leq 0.05$

4.5. DNA protection studies

When pUC18 plasmid DNA was exposed to Fenton's reagent, it caused conversion of DNA band from Form I (Native plasmid DNA) to Form II (single-stranded, nicked circular plasmid DNA) while in the case of calf thymus DNA protection study, the exposure of native DNA to Fenton's reaction, it caused fragmentation of DNA with disappearance of DNA bands. It is clear from the results that the addition of KPE and KPF to reaction mixture protects DNA at the concentration of 50 µg/ml and 250 µg/ml in pUC18 and calf thymus DNA protection studies respectively by scavenging of the ·OH radicals generated by Fenton reaction (Fig. 5-7).

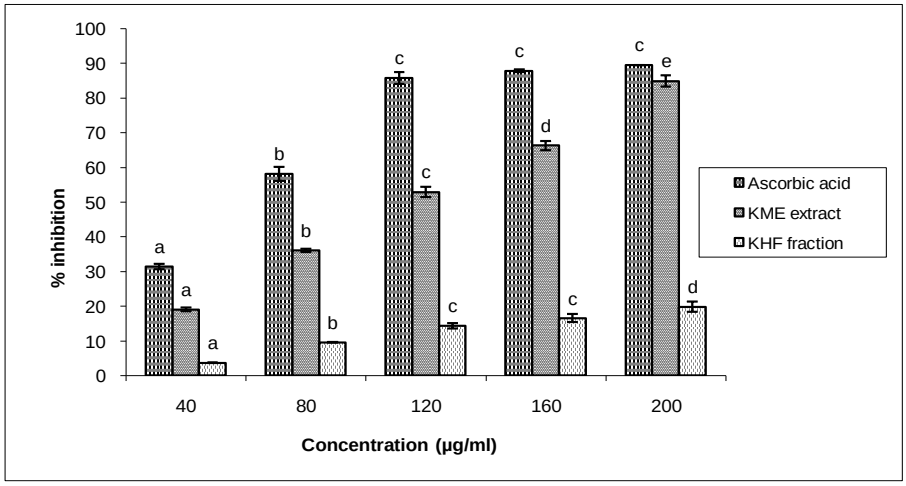


Figure 2. Scavenging effects of KPE and KPF of leaves and standard asorbic acid on DPPH radicals. Different letters (a,b,c,d &e) with in the same extract/fraction indicates that these concentrations are significantly different from each other at the level of $p \leq 0.05$

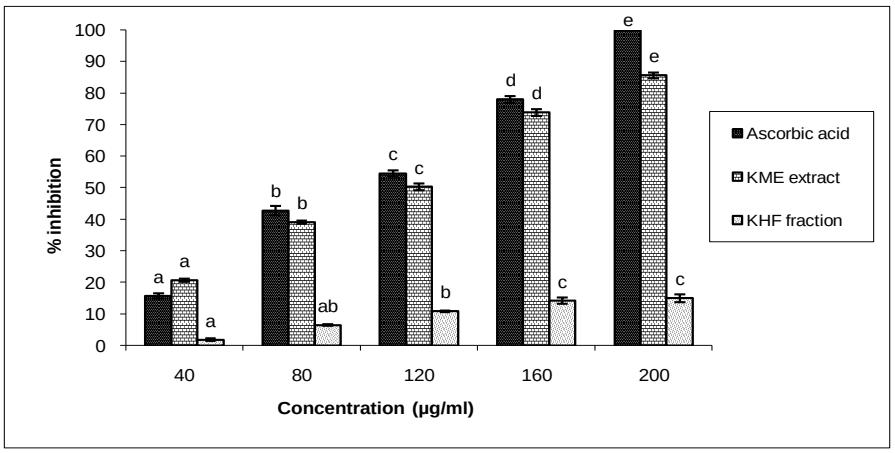


Figure 3. Reducing potential of KPE and KPF of leaves in comparision to standard asorbic acid. Different letters (a,b,c,d &e) with in the same extract/fraction indicates that these concentrations are significantly different from each other at the level of $p \leq 0.05$

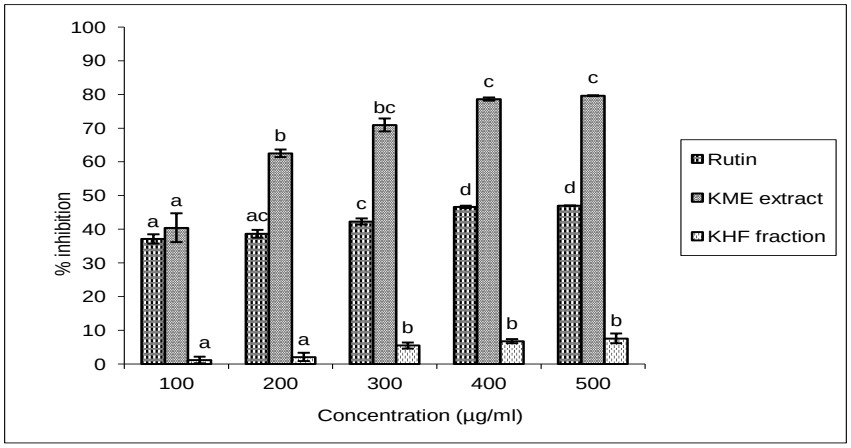


Figure 4. Scavenging effects of KPE and KPF of leaves and standard rutin on superoxide radicals. Different letters (a,b,c,d &e) with in the same extract/fraction indicates that these concentrations are significantly different from each other at the level of $p \leq 0.05$

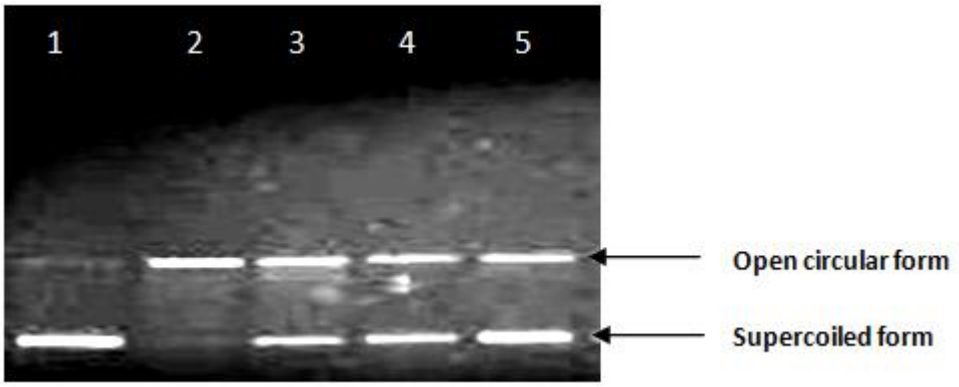


Figure 5. Effect of KPE and KPF on the protection of supercoiled DNA against hydroxyl radical generated by the H_2O_2. Lane 1: pUC18 DNA, Lane 2: pUC18 DNA + Fenton's reagent (DNA damage control), Lane 3: pUC18 DNA + Fenton's reagent + Gallic acid (Standard) (50µg/ml), Lane 4: pUC18 DNA + Fenton's reagent + KPE (50 µg/ml), Lane 5: pUC18 DNA + Fenton's reagent + KPF (50 µg/ml).

Figure 6. Effect of KPE on the protection of calf thymus DNA against hydroxyl radical generated by the H_2O_2. Lane 1: Calf thymus DNA, Lane 2: Calf thymus DNA + Fenton's reagent (DNA damage control), Lane 3: Calf thymus DNA + Fenton's reagent + Rutin (Standard) (250 µg/ml), Lane 4: Calf thymus DNA + Fenton's reagent + KPE (250 µg/ml)

Figure 7. Effect of KPF on the protection of calf thymus DNA against hydroxyl radical generated by the H_2O_2. Lane 1: Calf thymus DNA, Lane 2: Calf thymus DNA + Fenton's reagent (DNA damage control), Lane 3: Calf thymus DNA + Fenton's reagent + Rutin (Standard) (250 µg/ml), Lane 4: Calf thymus DNA + Fenton's reagent + KPF (250 µg/ml).

4.6. Phytochemical analysis

Total Phenolic Compounds and Total Flavonoid compounds of KPE was found to be 517.5 mg/g of GAE (Gallic acid equivalents) and 300 mg/g of RE (Rutin equivalents) respectively while that of KPF lacks phenolic and flavonoid constituents.

5. Discussion

ABTS and DPPH radicals are two chemically synthesized stable radicals with high sensitivity employed for evaluation of antioxidant ability of various pure compounds [2]. The ABTS assay is based on the reduction of the radical cation of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate)

($ABTS^{\cdot+}$) which has a characteristic long-wavelength absorption maxima at 734 nm by plant extracts. This assay involves the generation of the ABTS chromophore by the oxidation of ABTS with potassium persulfate. Both hydrophilic and lipophilic compounds can be tested by this method. The assay has been widely used in many recent studies related to detection of antioxidant property of plant extracts [34]. The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical to become colourless in the presence of plant extracts. The DPPH radical possess an odd electron which is responsible for the absorbance at 517 nm and also for visible purple color. When DPPH accepts an electron donated by an antioxidant test compound, the DPPH is decolorized which can be quantitatively measured from the decline in absorbance. Antioxidants are also believed to intercept the free radical chain of oxidation and donate hydrogen from the hydroxyl groups of polyphenols, thus forming a stable end-product, which does not begin or propagate further oxidation of lipids [33]. In our studies, the KPE showed excellent DPPH and ABTS radical scavenging potential (Fig. 1&2) and its phytochemical analysis showed a very high amount of phenolic/flavonoid content. The KPF exhibited very poor potential to inhibit both DPPH and ABTS radicals and showed the absence of both phenolic/flavonoid compounds.

The reduction of Fe^{3+} by antioxidant compounds is regarded as an indicator of electron-donating potential and chief mechanism of phenolic antioxidant action. The antioxidant compounds in test samples causes the reduction of Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) that can be monitored by measuring the formation of Perl's Prussian blue color at 700 nm [5]. Different studies indicated that electron donation capacity i.e reducing power has been related with antioxidant activity [6, 32]. The reducing capacity of various extract/fractions was evaluated using method by Oyaizu, 1986 [27]. In this assay, the yellow colour of the test solution changes to Prussian blue color depending on reducing power of antioxidant samples. Reducing power indicates compounds that are electron donors which can act as primary and secondary antioxidants [37]. The KPE showed very good reducing capability than KPF (Fig. 3). Higher reducing powers might be accredited to higher amounts of total phenolic and flavonoid [20]. Different studies have been indicated that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [30, 31].

Superoxide anion is biologically fatal and used by immune system against attacking microorganisms. The biological toxicity of superoxide is due to its ability to inactivate iron sulphur cluster containing enzymes, which are vital in wide variety of metabolic pathways, thus, liberating free iron in the cell, which undergo Fenton-chemistry and generate the highly reactive hydroxyl ions [7]. Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thus resulting in tissue damage [8]. Superoxide anions are also reported to cause direct lipid peroxidation [35]. Superoxide anion plays an important role in the formation of other reactive oxygen species such as singlet oxygen and $OH^{\cdot}$ which causes damage to various biomolecules [28]. The KPE had the best overall ability to scavenge superoxide anion (79.64%) and the KPF had low superoxide anion ($O^{2\bullet-}$) scavenging efficiency with only 7.6% inhibition of $O^{2\bullet-}$ production (Fig. 4). This shows that phenolic and flavonoids may be responsible for this excellent scavenging potential of KPE. Further many reports showed the isolation of various flavonoid compounds from the leaves of *K. paniculata* [24, 22].

DNA can be damaged by free radicals as reported by LIoyd and Phillips, 1999 [23]. Hydroxyl radical generated by Fenton's reagent attacks supercoiled pUC18 plasmid DNA and causes single stranded scission (resulting in nicked circular form II) or double stranded breaks (resulting in linear form III). However, in case of calf thymus DNA, hydroxyl radicals generated as result of Fenton chemistry, cause complete fragmentation of genomic DNA. The addition of KPE and KPF along with Fenton's reagent provide protection to plasmid DNA/genomic DNA and resulting in retention of native form indicating

KPF to possess certain non-phenolic genoprotective constituents. The effect of the extract/fractions was compared with standard antioxidant compounds such as gallic acid in plasmid DNA (Fig 5) and rutin in case of calf thymus DNA protection studies (Fig 6&7).

From the present study, it was concluded that KPE possessed good antioxidant activity than KPF, this may be due to the presence of polyphenolic compounds as these were absent in KPF. The study warrants further investigation for the isolation and characterization of these extracts for their eventual application in chemopreventive studies.

Acknowledgments

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