



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(12)60313-X ©2012 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

Efficacy of golden rain tree against free radicals and H₂O₂-induced damage to pUC18/calf thymus DNA

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ARTICLE INFO

Article history:

Received 25 June 2012

Received in revised form 25 July 2012

Accepted 7 August 2012

Available online 28 August 2012

Keywords:

Oxidative stress

DNA damage

Fenton's reagent

Koelreuteria paniculata Laxm

Genoprotective potential

Antioxidants

ABSTRACT

Objective: To investigate the antioxidant and genoprotective potential of the methanol extract (METH-KP) along with its hexane fraction (HEX-KP) from the leaves of *Koelreuteria paniculata* (*K. paniculata*) Laxm. **Methods:** The antioxidant potential was checked using metal chelation assay, ABTS, DPPH (2,2-diphenyl-2-picryl-hydrazyl), reducing power and superoxide anion radical scavenging assay, Folin-Ciocalteu reducing capacity (FCR assay), Total flavonoid content (TFC assay) and genoprotective activity against the DNA damage induced by Fenton's reagent using pUC18/calf thymus. **Results:** Results showed that radical scavenging activities of the both test extract/fraction revealed a concentration-dependent antiradical activity in all the assays. In Metal chelation assay, METH-KP and HEX-KP showed 39.04% and 32.51% of scavenging at highest tested concentrations. The METH-KP exhibited IC₅₀ 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 HEX-KP 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 METH-KP while the HEX-KP fraction lacks phenols. The METH-KP extract and HEX-KP fraction both showed DNA protective effect in Calf thymus/pUC18 DNA protection studies. **Conclusions:** The activity of METH-KP may be attributed to its polyphenolic constituents which needs further isolation of its active constituents which may lead to the development of novel drugs to combat cancer.

1. Introduction

Excessive production of reactive oxygen species in living systems can cause oxidation of biomolecules viz. proteins, lipids, carbohydrates etc. Free radicals also results in degradation of food stuffs. Generally, in living system there is a balance between oxidant and antioxidant activities. Imbalance of this oxidant and antioxidant system towards oxidant side results in harm to various functional tissues. Free radicals may cause structural changes in biomolecules[1,2]. Organisms are provided by nature with antioxidant defence system (including antioxidative enzymes and compounds) which protects them against the oxidative stress. However, when there is excessive generation of these radicals, body requires exogenous source of antioxidant compounds which can delay or inhibit

the initiation or propagation of oxidative chain reactions. Now a days, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgalate (PG) and tert-butyl hydroquinone (TBHQ), are used commercially as food additives. However, in recent years, BHA and BHT are under strict regulation about their use in food, because of toxicity[3,4]. Recently, research regarding the natural source of antioxidants have received much attention because they are more safer and efficient than synthetic compounds[5].

The crude extracts of several medicinal plants have been reported to demonstrate an antioxidant capacity as well as a protective effect against DNA damage induced by oxidative mutagens[6-11]. Plant extracts are the source of natural antioxidants that may serve as leads for the novel drugs development. Numerous anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been discovered which acts through an antioxidant mechanism as component of their activity[12]. Further, products of natural origin are more safe for consumption and act as substitutes for synthetic drugs.

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Financial assistance: No. 36-93/2009 (SR)

Since the past two decades, 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^[13–15]. *Koelreuteria paniculata* (*K. 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 and oxidative stress ameliorating potential of *K. paniculata* Laxm. extract (METH–KP) and *K. paniculata* Laxm. fraction (HEX–KP) of leaves.

2. Materials and methods

2.1. Chemicals

2,2–Diphenyl–1–picrylhydrazyl (DPPH), Ferric chloride, Nicotinamide adenine dinucleotide (NADH), phenazine methosulphate, nitrobluetetrazolium, ferrozine, 2,2–azinobis (3–ethylbenzothiazoline–6–sulfonate) (ABTS), BHT and L–Ascorbic acid were obtained from HiMedia Pvt. Ltd. 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., Bangalore, India. All other reagents were of analytical grade (AR).

2.2. Plant material

2.2.1. Collection of plant material

The leaves of the plant *K. paniculata* were collected from Botanical garden of Guru Nanak Dev University, Amritsar, Punjab. The specimen was identified and voucher specimen No. 0409/HRB was deposited in herbarium of the Department.

2.2.2. Extraction and isolation

The leaves were washed with running tap water to remove dust impurities and finally dried at 40 °C. 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 METH–KP (35 g). METH–KP 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 HEX–KP (7.32 g).

2.3. DNA protection assay

A DNA protection assay was performed using supercoiled pUC18 plasmid/calf thymus DNA with slight modifications^[16]. Plasmid/calf thymus DNA was incubated with Fenton's reagents (30 mM H₂O₂, 50 mM ascorbic acid and 80 mM FeCl₃) containing test sample and finally the 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).

2.4. Antioxidant assays

2.4.1. Metal chelation assay

The chelating activity of extract/fraction was measured as given by Dinis *et al*^[17] with little modifications. 1 mL of extract with different concentrations was mixed with 1 mL of methanol, and then the mixture was mixed with ferrous chloride (2 mM, 0.1 mL) and ferrozine (1 mM, 0.2 mL) for 10 min at room temperature. The absorbance was measured at 562 nm against a blank in which the extract was replaced by vehicle solvent. EDTA was used as standard reference.

The % age inhibition was calculated as:

$$\% \text{ Inhibition} = A_0 - A_1/A_0 \times 100$$

Where, A₀ is the absorbance of control, A₁ is the absorbance of reaction mixture.

2.4.2. ABTS.⁺ radical scavenging assay

ABTS.⁺ scavenging assay was carried out by the method given by Re *et al*^[18]. 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 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.

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

Where, A₀ is the absorbance of ABTS solution, A₁ is the absorbance of reaction mixture (containing test sample & ABTS solution).

2.4.3. DPPH–radical scavenging assay

DPPH scavenging activity was carried out by the method of Blois^[19] with slight modifications. Different concentrations (40–200 µg/mL) of test samples of *K. 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. After 30 min, 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.

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

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

2.4.4. Ferric ion reduction potential

Reducing potential of extract/fraction was determined using the method of Oyaizu^[20]. Different concentrations (40–200 $\mu\text{g/mL}$) of test samples of *K. 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 min. After incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added and kept for centrifugation at 3 000 rpm for 10 minutes. After centrifugation, 2.5 mL of supernatant was taken and to this, 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 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 in comparison to the standard (Ascorbic acid) was calculated using the formula:

$$\% \text{ Reducing power} = [1 - (1 - A_s/A_c) \times 100]$$

Where, A_c = absorbance of standard compound at maximum concentration tested, and A_s = absorbance of sample.

2.4.5. Superoxide anion radical scavenging assay

The measurement of superoxide anion scavenging activity was performed based on the method described by Nishikimi *et al*^[21] 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 $\mu\text{g/mL}$) 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.

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

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

2.5. Phytochemical analysis

2.5.1. Determination of total phenolic content

The total phenolic content (TPC) of the METH–KP/HEX–KP

was determined using Folin–Ciocalteu method of Yu *et al*^[22] employing gallic acid as standard. To 100 μL of test sample (100 $\mu\text{g/mL}$) 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) in mg/g of dry sample.

2.5.2. Determination of total flavonoid content

The method given by Kim *et al*^[23] 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 $\mu\text{g/mL}$ extract /fractions, 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 min. To this mixture 2 mL of NaOH was added and the final volume was raised to 10 mL. Finally absorbance was taken at 510 nm. The total flavonoid content was then expressed as rutin equivalents (RE) in mg/g of dry sample.

2.6. Statistical analysis

The experimental results were expressed as mean \pm 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 ($P \leq 0.05$).

3. Results

3.1. 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 METH–KP and HEX–KP to reaction mixture protects DNA at the concentration of 50 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ in pUC18 and calf thymus DNA protection studies respectively by scavenging of the $\cdot\text{OH}$ radicals generated by Fenton reaction (Figure 1–3).

3.2. Antioxidant studies

In Metal chelation assay, METH–KP and HEX–KP showed 39.04% and 32.51% of scavenging at highest tested concentrations (Table 2). However, reference standard EDTA showed 90% at the same concentration with IC_{50}

Table 1. Total phenolic/flavonoid contents in the METH-KP and HEX-KP of *K. paniculata*.

Extract/fraction	TPC (mg GAE/g dry wt. of extract)	TFC (mg RE/g dry wt. of extract)
METH-KP	517.5	300
HEX-KP	Nil	Nil

Table 2. Maximum percentage inhibition achieved by METH-KP and HEX-KP in different *in vitro* free radical scavenging assays.

S .No.	Assay	METH-KP (Maximum % inhibition)	HEX-KP (Maximum % inhibition)
1	Metal Chelation assay	39.04	32.51
2	ABTS. ⁺ assay	98.35	29.11
3	DPPH assay	84.93	19.86
4	Reducing power assay	85.63	15.00
5	Superoxide anion radical scavenging assay	79.64	7.60

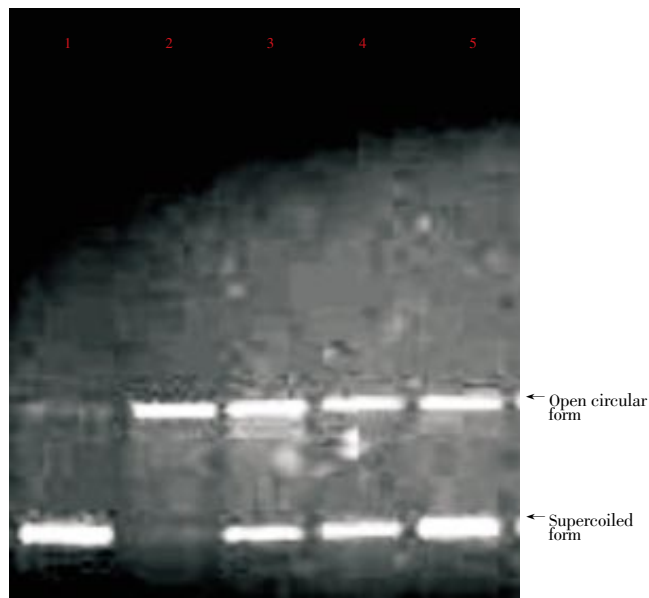


Figure 1. Effect of METH-KP and HEX-KP on the protection of supercoiled DNA against hydroxyl radical generated by the H₂O₂. Lane 1: pUC18 DNA, Lane 2: pUC18 DNA + Fenton's reagent (DNA damage control), Lane 3: pUC18 DNA + Fenton's reagent + Gallic acid (Standard) (50 μg/mL), Lane 4: pUC18 DNA + Fenton's reagent + METH-KP (50 μg/mL), Lane 5: pUC18 DNA + Fenton's reagent + HEX-KP (50 μg/mL).

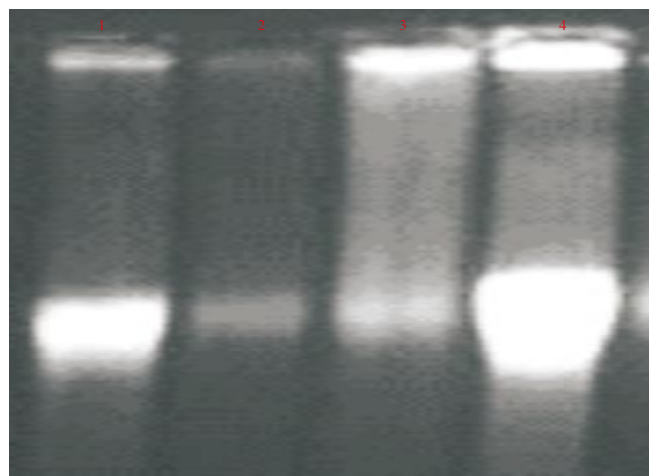


Figure 2. Effect of METH-KP on the protection of calf thymus DNA against hydroxyl radical generated by the H₂O₂. Lane 1: Calf thymus DNA, Lane 2: Calf thymus DNA + Fenton's reagent (DNA damage control), Lane 3: Calf thymus DNA + Fenton's reagent + Rutin (Standard) (250 μg/mL), Lane 4: Calf thymus DNA + Fenton's reagent + METH-KP (250 μg/mL).



Figure 3. Effect of HEX-KP on the protection of calf thymus DNA against hydroxyl radical generated by the H₂O₂. 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 + HEX-KP (250 μg/mL).

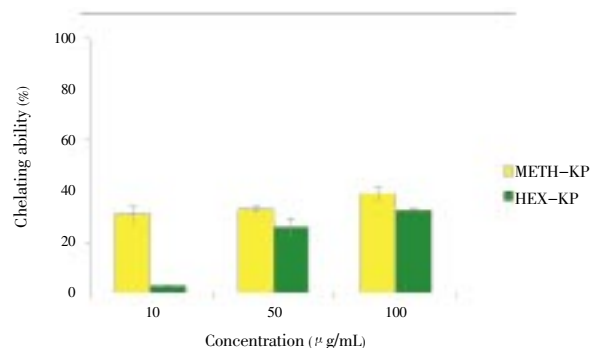


Figure 4. Metal chelating ability of extract/fraction of *K. paniculata* leaves.

of 23.26 $\mu\text{g/mL}$ (Figure 4). In ABTS assay, The METH–KP exhibited radical scavenging activity of 98.35% (IC_{50} of 54.54 $\mu\text{g/mL}$) while HEX–KP fraction showed 29.11% of inhibition at the concentration of 200 $\mu\text{g/mL}$ (Table 2). The standard compound BHT showed 51.6% inhibition at concentration of 200 $\mu\text{g/mL}$ (IC_{50} of 197.55 $\mu\text{g/mL}$) (Figure 5). The METH–KP extract showed promising free radical scavenging effect with inhibition 84.93% (IC_{50} of 115 $\mu\text{g/mL}$) while HEX–KP showed poor inhibition of DPPH radicals with inhibition of 19.86% concentration of 200 $\mu\text{g/mL}$ (Figure 6). The antioxidant standard compound ascorbic acid showed 89.53% inhibition concentration of 200 $\mu\text{g/mL}$ (IC_{50} of 55.88 $\mu\text{g/mL}$).

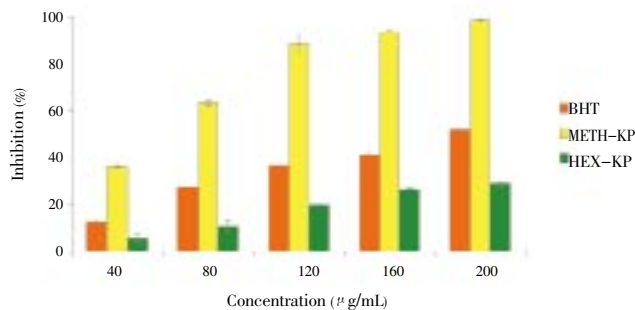


Figure 5. Scavenging effects of extract/fraction of *K. paniculata* leaves on ABTS radicals.

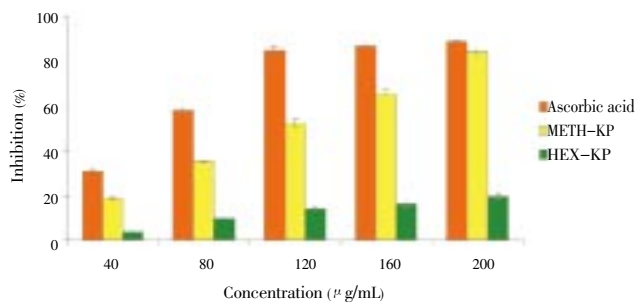


Figure 6. Scavenging effects of extract/fraction of *K. paniculata* leaves on DPPH radicals.

3.3. Phytochemical analysis

Total Phenolic Compounds and Total Flavonoid compounds of METH–KP was found to be 517.5 mg/g of GAE (Gallic acid equivalents) and 300 mg/g of RE (Rutin equivalents) respectively. HEX–KP fraction lacks phenolic and flavonoid constituents (Table 1).

4. Discussion

Transition metals (iron and copper) in the human body are responsible for various enzymes and for some protein activities involved in cellular respiration, transport of oxygen and redox reactions. Transition metals act as powerful catalysts of autoxidation reactions such as during conversion of H_2O_2 to $\text{HO}\cdot$ in the Fenton's reaction's, in conversion of alkyl peroxides to the highly reactive alkoxy and hydroxyl

The reducing capacity of a compound Fe^{3+} /ferricyanide complex to ferrous form may serve as indicator of its antioxidant capacity. At the concentration of 200 $\mu\text{g/mL}$, the METH–KP and HEX–KP exhibited 85.63% and 15.00% of inhibition in compared to standard ascorbic acid (Figure 7). The superoxide radical activity of extract/fraction was measured by non–enzymatic PMS–NADH system. The percent inhibition of superoxide anion radical generation by METH–KP and HEX–KP was found to be 79.64% and 7.60% at the concentration of 500 $\mu\text{g/mL}$ (Table 2). The results were compared with rutin (Figure 8).

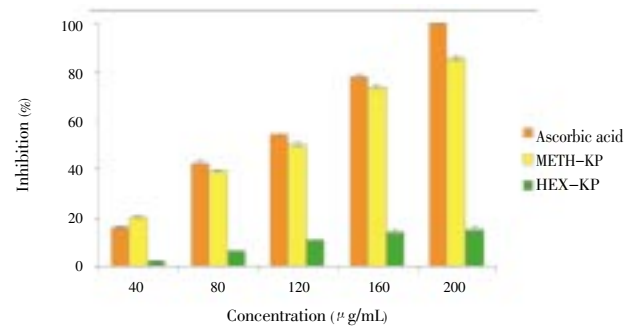


Figure 7. Reducing potential of different concentrations of extract/fraction of *K. paniculata* leaves in comparison to ascorbic acid (standard).

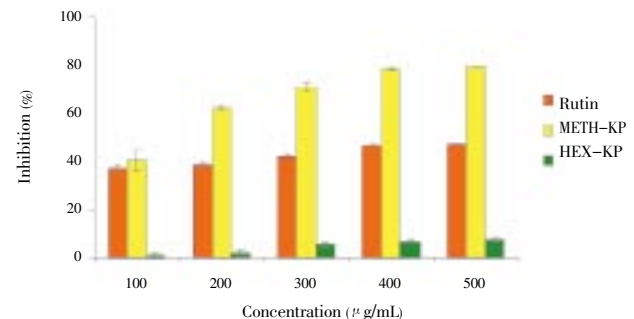


Figure 8. Scavenging effects of extract/fraction of *K. paniculata* leaves on superoxide radicals.

radicals etc[12]. Our results showed that both samples possess metal chelating activity showing their ability to chelate Fe(II) . Minimizing ferrous ion (Fe^{2+}) may provide protection against oxidative damage by inhibiting production of ROS and molecular damage. ABTS^+ scavenging assay is used for both lipophilic and hydrophilic test samples. ABTS^+ radicals are more reactive than DPPH radicals because the reaction with DPPH radical involves only hydrogen atom transfer while in case of ABTS^+ radicals, the reaction involves the electron transfer along with proton donation. The ABTS^+ cations are generated by oxidation of ABTS with $\text{K}_2\text{S}_2\text{O}_8$ [3,12]. ABTS and DPPH radicals are two chemically synthesized radicals with high sensitivity employed for evaluation of antioxidant ability of various pure compounds[12]. The ABTS assay is based on the reduction of the radical cation of 2,2–azinobis (3–ethylbenzothiazoline–6–sulfonate) (ABTS^+) 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. The assay has been widely used in many recent studies related to detection of antioxidant property of plant extracts. 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^[12,24]. 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^[25]. In our studies, the METH–KP showed excellent DPPH and ABTS radical scavenging potential and its phytochemical analysis showed a very high amount of phenolic/flavonoid content. The HEX–KP 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. Different studies indicated that electron donation capacity i.e reducing power has been related with antioxidant activity^[26–28]. In reducing power 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^[12]. The METH–KP showed very good reducing capability than HEX–KP. Higher reducing powers might be accredited to higher amounts of total phenolic and flavonoid. 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^[29–31].

Superoxide anion is biologically fatal and used by immune system against attacking microorganisms. Superoxide anions possess ability to inactivate iron sulphur cluster containing enzymes, which are vital in wide variety of metabolic pathways, thus, releasing free iron in the cell, which undergo Fenton-chemistry and generate the highly reactive hydroxyl ions. Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thus resulting in tissue damage. Superoxide anions are also reported to cause direct lipid peroxidation. Superoxide anion plays an important role in the formation of other reactive oxygen species such as singlet oxygen and OH^\bullet which causes damage to various biomolecules^[12,32,33]. The METH–KP showed the best overall ability to scavenge superoxide anion (79.64%) and the HEX–

KP showed low superoxide anion ($\text{O}_2^{\bullet-}$) scavenging efficiency with only 7.6% inhibition of $\text{O}_2^{\bullet-}$ production. This shows that phenolic and flavonoids may be responsible for this excellent scavenging potential of METH–KP. Further many reports showed the isolation and bioactivities of various fractions/flavonoid compounds from the leaves of *K. paniculata*^[7,10,11,34,35].

DNA can be damaged by free radicals as reported by different workers^[7,10,11]. 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 METH–KP and HEX–KP along with Fenton's reagent provide protection to plasmid DNA/genomic DNA and resulting in retention of native form indicating HEX–KP to possess certain non-phenolic genoprotective constituents. The effect of the extract/fraction was compared with standard antioxidant compounds such as gallic acid in plasmid DNA and rutin in case of calf thymus DNA protection studies.

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

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are thankful to the Council of Scientific and Industrial Research (CSIR) [38(1265)/10/EMR–II], New Delhi and University Grant Commission (UGC) [F. No. 36–93/2009 (SR)], New Delhi for providing for financial assistance and to the Department of Botanical and Environmental Sciences for providing necessary research facilities.

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