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Full Length Research Paper

Antioxidant, antimicrobial and cytotoxicity studies of *Russelia equisetiformis*

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In the present study of *Russelia equisetiformis* belonging to the family scrophulariaceae, leaves were evaluated for antioxidant, antimicrobial and cytotoxicity studies. The leaves were extracted with absolute methanol and further fractionated by solvent-solvent extraction method with increasing polarity based absolute solvents, that is, *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The qualitative analysis of phytochemicals such as alkaloids, flavonoids, saponins, tannins, steroids and terpenoids were carried out. The total phenolics and flavonoids content were analyzed by spectroscopic technique. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging and linoleic acid oxidation assays were carried out. The inhibitory concentration (IC_{50}) and percentage inhibition by linoleic acid oxidation was found to be in the range of 9.96 to 89.79 and 39.46 to 90.69, respectively. The antioxidant activity was also studied to evaluate the protective effect of plant by H_2O_2 induced oxidative damage in plasmid pBR322 DNA and found that it protected the DNA. The potential of the extract and fractions against selected bacterial (*Pasturella multocida, Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*) and fungal strains (*Aspergillus niger, Aspergillus flavus, Alternaria alternate* and *Rhizopus solani*) was evaluated in the present study. The plant extract and fractions were assayed against human red blood cells (RBCs) and the percentage lysis was found to be in the range of 1.89 to 4.96 %.

Key words: *Russelia equisetiformis*, antimicrobial, deoxyribonucleic acid (DNA), haemolytic activity, inhibitory concentration(IC_{50}).

INTRODUCTION

The use of medicinal plants as the first medicine is a universal phenomenon (Serrentino, 1991). Plants are known to be the richest source of natural antioxidants and antimicrobial compounds (Albayrak et al., 2010; Farombi, 2003). The importance of plants as remedy for diseases has necessitated a proper evaluation of plants by biological and phytochemical studies.

Russelia equisetiformis (Firecracker) is an evergreen, perennial shrub with attractive looking, plants feature trailing, green stems and tubular red blossoms. When their cultivation requirements are met, firecracker plants produce their distinguishing blossoms. Firecracker plants are easily propagated from rooted cuttings. *R. equisetiformis* is a shrub belonging to the family scrophulariaceae. It grows up to four feet high with red flowers, and much reduced leaves. *R. equisetiformis* is traditionally used as a medicinal plant and considered to have anti-inflammatory, analgesic and membrane stabilizing properties (Awe et al., 2009).

Many plants that have potential to be used as natural remedy against diseases were described by Erdemgil

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et al. (2007). The studies of the antioxidant potential of phytochemical constituents found in plants may also be necessary because of their medicinal properties (Zhu et al., 2004). The antioxidants are chemical constituents that can delay the oxidation of lipids containing products. The antioxidant action of phytochemicals is mostly due to their redox property, which take part in very important role for capturing free radicals and scavenging of oxygen (Moosmann and Behl, 1999). The antimicrobial compounds inhibit the growth of microorganisms, such as fungi, bacteria or protozoan. Antimicrobial drugs prevent the growth of microbes. The antimicrobial and antioxidant actions of plant extract have been formed from the beginning of many applications including preservation of food by using natural sources (Bozin et al., 2006). Keeping in view, the importance of antioxidant and antimicrobial activity of present study was conducted to evaluate the potential of *R. equisetiformis* (firecracker) as antioxidant and antimicrobial agent.

MATERIALS AND METHODS

R. equisetiformis (scrophulariaceae) leaves were collected from the Botanical Garden University of Agriculture Faisalabad, Pakistan. The plant further identified and authenticated by the Dr. Mansoor Hameed (Taxonomist), Department of Botany, University of Agriculture, Faisalabad Pakistan. A voucher specimen (4203) was deposited in collection/herbarium Department of Botany University of Agriculture Faisalabad. After collection, plant leaves were washed, shade dried and ground. The plant material 1 kg was extracted thrice with absolute methanol by dipping for seven days. The methanol extract concentrated to dryness under reduced pressure using rotary evaporator. The methanol extract was further fractioned using solvent extraction method with different polarity based absolute solvents such as *n*-hexane, chloroform, ethyl acetate and *n*-butanol. After fractionation samples, concentrated to dryness under reduced pressure using rotary evaporator and stored in a refrigerator at -4°C, until used for analysis.

Phytochemical analysis

Phytochemical analysis was carried out by using the standard procedures to indentify the constituents qualitatively in extract/fractions and quantitatively in dried leaves as method described by Edeoga et al. (2005).

Determination of total phenolics content

The total phenolics content were determined using folin ciocalteau phenol reagent as process described by Chaovanalikit and Wrolstad (2004). 50 mg of the extract and fractions were dissolved with 0.5 ml folin ciocalteau phenol reagent and 7.5 ml of distilled water. The mixture was incubated at room temperature for 10 min. After incubation, 1.5 ml (20%) of Na_2CO_3 (w\v) was added to the mixture. The mixture was heated on water bath for 20 min at 40°C and cooled immediately in an ice bath, and the absorbance noted at 755 nm. The amount of total phenolic contents were calculated using calibration curve for gallic acid (20 to 200 mg/L, $R^2 = 0.998$). The results were expressed as gallic acid equivalent (GAE) per dry matter. The experiments were performed in triplicate and their values averaged.

Determination of total flavonoid contents

Spectrophotometric method described by Dewanto et al. (2002) was used to determine the total flavonoid contents with minor modifications. The extract containing 0.01 g/ml of dry matter was dissolved in flask with an addition of 5 ml of distilled water. In that flask, 0.3 ml of 5% $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ was added, and after 5 min, 2 ml of 1 M NaOH was added. The solution was mixed well in the flask. The absorbance was noted at 510 nm. Total flavonoid contents were expressed as catechin equivalent (CE) per g of dry matter.

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH free radical scavenging activity of sample was determined using method described by Iqbal et al. (2005). The sample solution of extract and fractions having concentration ranging from 0.1 to 100 µg/ml was made in methanol with dilution method. 1 ml of the sample solution was mixed with 2.5 ml of DPPH at a concentration of 0.025 g/L, after 30 min the absorbance was noted at 515 nm. The inhibitory concentration (IC_{50}) values were calculated from the plot of concentration against percentage scavenging. Three replicates were recorded for each sample. The percentage scavenging by DPPH was calculated from the following equation:

$$\text{Scavenging (\%)} = 100 \times (A_{blank} - A_{sample}) / A_{blank}$$

Percentage inhibition of linoleic acid oxidation

The antioxidant activities of extract and fractions were also calculated in terms of measurement of percentage inhibition of per oxidation in the linoleic acid system using method described by Iqbal et al. (2005) and Yen et al. (2000). Synthetic antioxidant butylated hydroxytoluene (BHT) at 200 mg/L was used as a positive control. In the sample, the maximum oxidation level was noted at 360 h (15 days). The sample that contained no antioxidant component was used as a blank. The absorbance of samples was noted at 500 nm using a spectrophotometer. Percentage inhibition of linoleic acid oxidation was determined by the following equation:

Inhibition (%) = [100 - (Absorbance increase of sample at 360 h /Absorbance increase of control at 360 h) × 100]

Determination of reducing power

The reducing power of the extract and fractions was determined according to the procedure described by Yen et al. (2000). The plant extract and fractions containing 2.5 to 10.0 mg/ml of dry matter was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%); the mixture was incubated at 50°C for 20 min. Then 5 ml of 10% trichloroacetic acid added, centrifuged at 980 x*g* for 10 min at 5°C in refrigerated centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0 ml of distilled water. 1 ml of 0.1% ferric chloride was added and the absorbance noted at 700 nm.

DNA protection assay

The antioxidant activity of the methanol extract showing better antioxidant activity was put forward for DNA protection assay using method described with some modification by Kalpana et al. (2009).

Table 1. Qualitative phytochemical screening of *Russelia equisetiformis* leaves extract and fractions.

Name of phytochemical	Methanol	*n*-hexane	Chloroform	Ethyl acetate	*n*-butanol	Aqueous
Alkaloids	+	+	+	+	+	+
Steroids	+	+	-	+	-	+
Flavonoids	+	-	+	+	+	+
Tannins	+	-	+	+	+	+
Terpenoids	+	+	+	+	-	-
Saponins	+	-	-	+	+	+

Table 2. Percentage yield, total phenolics content (TPC), total flavonoids content (TFC) and antioxidant potential by *R. equisetiformis.*

Extract and fractions	Percentage yield of extract and fractions (g/100 g)	TPC (mg/g) GAE of dry matter	TFC (mg/g) CE of dry matter	DPPH (IC_{50}) (µg/ml)	% Inhibition of oxidation in linoleic acid
Aqueous	2.74± 0.03^{e}	3.14± 0.03^{e}	1.77 ± 0.01^{d}	65.21 ± 0.54^{b}	44.96 ± 0.35^{e}
n-butanol	3.64± 0.02^{d}	3.75 ± 0.04^{c}	2.89 ± 0.02^{c}	47.56 ± 0.35^{c}	51.16 ± 0.61^{d}
Chloroform	4.86± 0.03^{c}	2.69 ± 0.03^{d}	1.86 ± 0.01^{d}	44.18 ± 0.41^{d}	45.35 ± 0.52^{e}
Ethyl acetate	7.53± 0.05^{b}	5.11 ± 0.05^{b}	3.49 ± 0.04^{b}	36.21 ± 0.31^{e}	63.95 ± 0.45^{c}
n-hexane	2.41± 0.02^{f}	1.82 ± 0.02^{f}	0.72 ± 0.02^{e}	89.79 ± 0.92^{a}	39.46± 0.31^{f}
Methanol	23.50 ± 0.21^{a}	16.91 ± 0.06^{a}	14.56 ± 0.03^{a}	15.37 ± 0.12^{f}	76.74 ± 0.89^{b}
BHT	--	--	--	9.96 ± 0.08^{g}	90.69 ± 1.02^{a}

The results are the average of triplicate samples (n = 3) ± S.D, ($p < 0.05$). The superscript letters represent the significant differences.

Antimicrobial activity

In order to evaluate the antimicrobial activity of selected bacterial, that is, *Pasturella multocida* (locally isolated), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (JS 2004) and *Staphylococcus aureus* (API staph tac 6736153) and fungal strains, that is, *Aspergillus niger* (ATTC 10595), *Aspergillus flavus* (ATCC 32612), *Alternaria alternate* (ATCC 20084) and *Rhizopus solani* (locally isolated) by using plant extract and fractions analyzed by disc diffusion method (NCCLS, 1997), minimum inhibitory concentration (MIC) with some modification using method described by Sarker et al. (2007).

Haemolytic activity for cytotoxicity assay

To evaluate the cytotoxicity of the extract and fractions, haemolytic activity was studied using a method described Powell et al. (2000).

Statistical analysis

The experiments were carried out in triplicate and statistical analysis of the data was performed by analysis of variance, using STATISTICA 5.5. The probability value $p \leq 0.05$ was considered to denote a statistically significance evaluation. All data were presented as mean values and standard deviation (SD).

RESULTS AND DISCUSSION

Yield of extracts

The yield of plant extract and fraction was found in the range of 2.41 to 23.50 g/100 g of dry plant. The maximum yield value was observed with absolute methanol while minimum value for the *n*-hexane fraction. The yield of extracts in various solvents is summarized in Table 2. Our findings of extraction of plant leaves are in accordance with earlier reports (Sultana et al., 2007) showing a good efficiency of methanol solvent for extraction phytochemical constituents from plant sources.

Phytochemical study

Phytochemicals such as flavonoids, saponins, tannins, steroids, alkaloids and terpenoids found in *R. equisetiformis* were evaluated qualitatively, and is shown in Table 1. These constituents were present in plant extract and fractions except flavonoids, tannins and saponins which were absent in the *n*-hexane fraction, while terpenoids and steroids were absent in *n*-butanol fraction. The phytochemicals were known to show these biological activities by Sofowora (1993). On the other hand, flavonoids, polyphenol and terpenoids have been identified from previous studies in plants as detailed by Ojewole (2005) and various bioactive compounds have been isolated from extracts of the plant as discussed by Yamagishi et al. (1989). As per literature review the crude percentage of secondary metabolites such as alkaloids, phenols, tannins, flavonoids and saponins in Nigerian plants were presented by Edeoga et al. (2005), and almost same trend was observed in the plant used in the present work.

Total phenolics and flavonoids content

The total phenolics content analyzed in *R. equisetiformis* in methanol extract were found to be the highest 16.91 mg/g, while the lowest TPC was observed in *n*-hexane (1.82 mg/g) gallic acid equivalent (GAE) of dry matter (Table 2). The total flavonoids content in *R. equisetiformis* extract and fractions of different solvent were also evaluated and summarized in Table 2. The methanol extract has the highest amount of TPC and TFC compared to other fractions. The less concentration of phenolics and flavonoids in aqueous extract might be attributed to the polyphenol oxidase enzyme, which degrade polyphenols in water extracts, whereas in methanol solvent they are inactive (Tiwari et al., 2011). Additionally methanol was found easier to penetrate than the cellular membrane to extract the intracellular ingredients from the plant material. The methanol has been reported as useful solvent to extract the phenolics and flavonoid components discussed by Siddhuraju and Becker (2003), confirming the present findings.

DPPH free radical scavenging

The percentage scavenging potential of methanol extract and fractions was determined by DPPH radical, and is summarized in Table 2. The greater IC_{50} value 89.79 µg/ml was of *n*-hexane fraction and least value 15.37 µg/ml was shown by methanol extract. The least value of IC_{50} represents the better antioxidant and greater value for less antioxidant activity. In this assay, the synthetic antioxidant butylted hydroxytoluene was used as positive control. The DPPH radical is usually used for the evaluation of antioxidant potential.

The DPPH radical has deep violet color when it reacts with hydrogen of donor species such as phenolics and upon receiving a proton it loses its color and becomes yellow (Hussain et al., 2008). In this assay, BHT was used as positive control. However, IC_{50} of BHT was observed least than methanol extract and fractions (Table 2). The results showed that the plant extract and fractions might have an antioxidant potential.

Percentage inhibition of linoleic acid oxidation

The findings of present research work showed percentage inhibitions of *R. equisetiformis* extract and fractions (Table 2). The extract and fractions showed percentage inhibition ranging between 39.53 to 90.69%. The highest antioxidant activity in terms of percentage inhibition was observed in methanol extract. The *n*-hexane fraction showed minimum inhibition. Whereas BHT used as a positive control showed 90.69% inhibition. These results suggested that the *R. equisetiformis* may be used for the lowering of lipid oxidation processes. The highest percentage inhibition in linoleic acid oxidation as an antioxidant activity was observed by methanol extract (Table 2). The highest level inhibition % of oxidation offered by the methanol extract might be due to the presence of greater concentration of TPC and TFC for this reason it could exhibit higher antioxidant activity. The *n*-hexane fraction showed minimum inhibition in the present investigation. The lower level of % inhibition in *n*-hexane extract can be explained by the lower concentration of phenolics as the *n*-hexane is a less polar solvent. As per literature review the % inhibition by plant extract was up to 90% as reported by Iqbal et al. (2005) and the results are comparable with our study; some variations may be due to the solvent used or the extraction procedure adopted. These results suggested that *R. equisetiformis* may be used for lowering of lipid oxidation processes.

Reducing power

The reducing power of the samples measured is summarized in Figure 1. The increase in antioxidant activity when the concentration of extract increased was observed by noting the absorbance spectrophotometrically. Increase in antioxidant potential when the concentration of extract and fractions increased was observed because an absorbance increased, showing the reducing power of extract and fractions. Of these, the highest was observed in the absolute methanol extract, which is in agreement with our findings that the extract has higher values of TPC, TFC, radical scavenging ability and percentage inhibition in linoleic acid oxidation. Almost the same trend was observed in work carried out with other plant extract by Sultana et al. (2009). The reducing power of phytochemical constituents may be associated with the antioxidant potential (Siddhuraju and Becker, 2003). Therefore, reducing power assessment may be taken as an important parameter for the evaluation of antioxidant activity of plant extracts.

Protection to DNA damaging

The results in Figure 2 showed the changes in the level of protection of plasmid pBR322 DNA by H_2O_2 induced damage when treated with different concentrations of *R. equisetiformis* methanol extract to evaluate the antioxidant activity. The methanol extract showed the highest antioxidant activity, so it proceeded for the evaluation of protection to DNA damaging. It was observed that the protection occurred when concentration of extract increased. In the lane (A) plasmid pBR322 DNA present without any treatment that might be due to dominant super coiled form. While lane (C) contain pBR322 DNA that was exposed to 4 µl H_2O_2 that caused damage in plasmid pBR322 DNA, in this lane the DNA and H_2O_2 as the result damaging of DNA strand occurred that might convert the super coiled form of pBR322 DNA into open linear form. The lane (B) showed the 1 kb DNA ladder. In

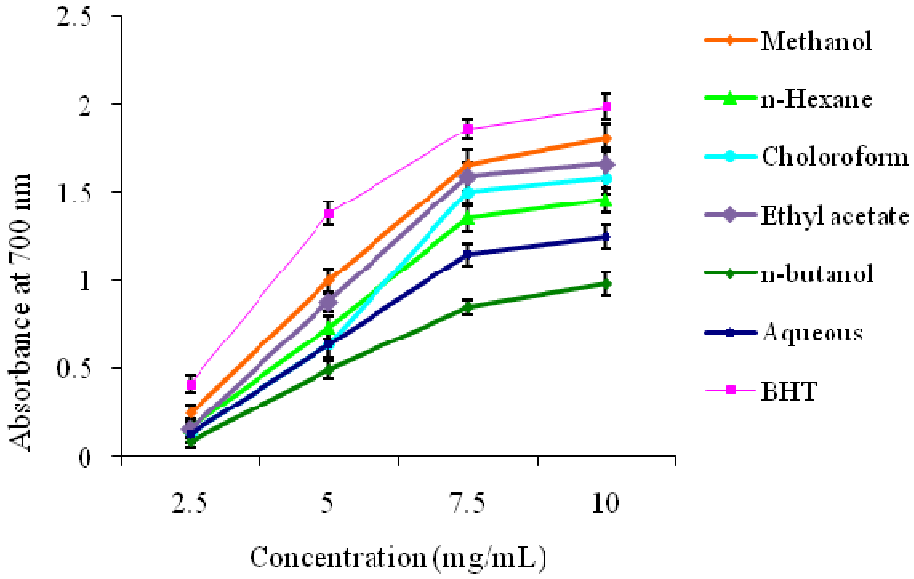

Figure 1. Reducing power of extract and fractions of *R. equisetiformis.*

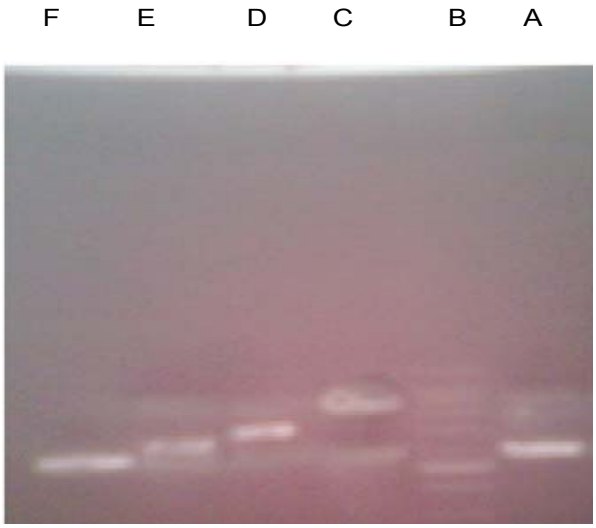

Figure 2. Electropherogram showing DNA protection effect by methanol extract with H_2O_2 induced oxidative damage on pBR322 DNA. Lane A = Plasmid pBR322 DNA without treatment (Super coiled); lane B = Plasmid pBR322 DNA treated with H_2O_2 (Open circular); lane C = 1 Kb DNA ladder; lane D = Plasmid pBR322 DNA treated with methanol extract (10 µg/ml) + H_2O_2; lane E = Plasmid pBR322 DNA treated with methanol extract (100 µg/ml) + H_2O_2; lane F = Plasmid pBR322 DNA treated with methanol extract (1000 µg/ml) + H_2O_2.

the lane (D to F) 5 µl (10, 100 and 1000 µg/mL) respectively of *R. equisetiformis* methanol extract was added in pBR322 DNA to observe their protective effect. The *R. equisetiformis* extract protected H_2O_2 induced strand breaks in plasmid pBR322 DNA in a concentration dependent manner. Figure 2 showed that the *R. equisetiformis* methanol extract at 1000 µg/mL protected the DNA; it may have converted the open circular form (damaged) of plasmid pBR322 DNA which was an indication of damage supercoiled form (protected). The

Table 3. Antimicrobial activity in terms of inhibition zone and by *R. equisetiformis* extract and fractions against selected bacterial and fungal strains.

Extract and fractions	Bacterial strains				Fungal strains			
	B. subtilis	***P. multocida***	***S. aureus***	***E. coli***	***A. flavus***	***A. niger***	***R. solani***	***A. alternata***
Inhibition zones (mm) by disc diffusion assay								
Methanol	15.35 ± 0.12^{b}	16.37 ± 0.12^{b}	18.35 ± 0.12^{b}	14.35 ± 0.12^{b}	13.23 ± 0.12^{b}	14.35 ± 0.12^{b}	15.33 ± 0.12^{b}	12.33 ± 0.12^{b}
n-butanol	11.23 ± 0.23^{e}	10.11 ± 0.09^{f}	N.D.*^{g}	9.83 ± 0.07^{e}	9.23 ± 0.09^{f}	8.66 ± 0.06^{d}	N.D.^{g}	8.84 ± 0.07^{d}
Chloroform	13.51 ± 0.24^{c}	12.23 ± 0.13^{d}	13.23 ± 0.12^{d}	10.36 ± 0.09^{d}	11.46 ± 0.09^{d}	N.D^{e}	11.15 ± 0.09^{d}	7.86 ± 0.06^{e}
Ethyl acetate	13.92 ± 0.12^{c}	13.23 ± 0.11^{c}	14.23 ± 0.10^{c}	13.36 ± 0.01^{c}	12.56 ± 0.12^{c}	11.42 ± 0.12^{c}	12.23 ± 0.12^{c}	11.36 ± 0.11^{c}
n-hexane	10.64 ± 0.16^{f}	9.51 ± 0.08^{g}	10.46 ± 0.08^{f}	9.42 ± 0.07^{e}	N.D.^{g}	7.83 ± 0.06^{d}	8.46 ± 0.07^{f}	N.D.^{f}
Aqueous	12.42 ± 0.09^{d}	11.30 ± 0.07^{e}	12.37 ± 0.13^{e}	N.D.^{f}	10.45 ± 0.02^{e}	N.D.^{e}	10.34± 0.08^{e}	N.D.^{f}
Rifampicine/ Fluconazole	29.61 ± 0.25^{a}	31.10 ± 0.25^{a}	28.72 ± 0.21^{a}	22.62 ± 0.21^{a}	27.53 ± 0.23^{a}	29.14 ± 0.24^{a}	24.74 ± 0.2^{a}	21.66 ± 0.15^{a}
Minimum inhibitory concentration (mg/ml)								
Methanol	5.44 ± 0.04^{f}	4.45 ± 0.03^{f}	3.45 ± 0.02^{e}	7.37 ± 0.05^{e}	8.20 ± 0.07^{e}	6.85 ± 0.05^{d}	5.51 ± 0.04^{e}	9.63 ± 0.08^{d}
n- butanol	27.38 ± 0.24^{d}	26.31 ± 0.19^{d}	N.D.^{f}	24.48 ± 0.14^{c}	30.72 ± 0.35^{c}	29.36 ± 0.21^{b}	N.D.^{g}	27.41 ± 0.21^{b}
Chloroform	31.32 ± 0.28^{c}	33.37 ± 0.27^{c}	25.32 ± 0.21^{c}	35.76 ± 0.26^{b}	33.85 ± 0.25^{b}	N.D.^{f}	27.54 ± 0.21^{c}	37.52 ± 0.31^{a}
Ethyl acetate	13.96 ± 0.14^{e}	12.34 ± 0.10^{e}	10.21 ± 0.01^{d}	14.45 ± 0.12^{d}	20.46 ± 0.21^{d}	18.56 ± 0.15^{c}	15.40 ± 0.12^{d}	24.43 ± 0.21^{c}
n -hexane	99.74 ± 1.03^{a}	95.35 ± 1.02^{a}	90.28 ±1.02^{a}	103.41 ± 1.02^{a}	N.D.^{g}	99.82 ± 1.13^{a}	96.27 ± 1.02^{a}	N.D.^{f}
Aqueous	80.26 ± 0.92^{b}	61.58 ± 0.92^{b}	76.36 ± 0.85^{b}	N.D.^{g}	89.23 ± 0.96^{a}	N.D.^{f}	78.36 ± 0.68^{b}	N.D.^{f}
Rifampicine/Fluconazole	3.43 ± 0.03^{g}	2.48 ± 0.01^{g}	1.45 ± 0.01^{e}	5.37 ± 0.04^{f}	5.51 ± 0.04^{f}	3.85 ± 0.02^{e}	2.72 ± 0.02^{f}	7.53 ± 0.06^{e}

*N.D. = Not detected; the values are the average of triplicate samples (n = 3) ± S.D., ($p < 0.05$); the superscript letters represent the significant differences.

protective effect observed by methanol extract may be due to higher concentration of TPC and TFC, and antioxidant activity and their antioxidant activity. The result was comparable with the other antioxidant assays carried out in present work. Thus, the *R. equisetiformis* methanolic extract may be a valuable antioxidant that protected plasmid pBR322 DNA from free radical induced oxidative damage. Apart from lipid oxidation, reactive oxygen species may causedamage to the cellular genetic material.

The protective effect of *R. equisetiformis* methanol extract on DNA can also be explained by its ability to scavenge reactive oxygen species due to its property as an antioxidant. By comparing with other results, there was an explanation for the protective effect which was the direct interaction of chemical constituents with DNA (Yoshikawa et al., 2006).

Antimicrobial activity

The antimicrobial assay of the methanol extract and fractions against selected microorganisms carried out were presented in Table 3. The plant leaves extract and fractions showed significant antimicrobial potential against most of the fungal and bacterial strains ($p < 0.05$). The findings from the disc diffusion method, followed by measurement of the minimum inhibitory concentration (MIC), indicated that absolute methanol extract showed good potential against bacterial strains *S. aureus,* showing the highest inhibition zones (18.35 mm) with lowest MIC values (3.45 mg/ml), while least inhibition zones by absolute methanol extract were observed for *E. coli* (14.35 mm) with MIC (7.37 mg/ml). The *n*-hexane fraction showed less activity against all the tested strains, that is, the zone of inhibition (mm), MIC (mg/ml) against *B. subtilis* (10.64 mm, 99.74 mg/ml), *S. aureus* (10.46 mm, 90.21 mg/ml), *P. multocida* (9.41 mm, 95.35 mg/ml), *E. coli* (9.42 mm, 103.40 mg/ml), respectively. No activity observed by n-butanol extract against *S.*

aureus, aqueous fraction against *E. coli*. The results indicated that absolute methanol extract showed good activity against fungal strains *R. solani,* showing the highest inhibition zones (15.33 mm) and the lowest MIC values (5.51 mg/ml), while against *A. alternata* fungal strain showed least inhibition zones (12.33 mm) with MIC (9.63 mg/ml). The *n*-hexane fraction showed least activity against all the tested strains, that is, the zone of inhibition (mm) and MIC (mg/ml), that is, against *A. alternata* (no activity), *R. solani* (8.46 mm, 96.27 mg/ml) and *A. niger* (7.82 mm, 99.8 mg/ml), respectively. *A. flavus* showed no activity in zone of inhibition and MIC by *n*-hexane fraction. There was observed no activity by *n*-butanol fraction against *R. solani*, chloroform fraction against *A. niger*, *n*-hexane fraction against *A. flavus* fungal strain. It was earlier reported that the phytochemical constituents found in plants active against microorganisms are aromatic or saturated organic compounds, and they are most often obtained through methanol extraction (Cowan, 1999). The rifampicine and fluconazole were used as positive control for bacterial and fungal strains respectively. The standard showed higher activity on the organisms than the tested extract and fractions (Table 3). The standard antibiotics were refined industrial products so their activity was more as compared to crude extract and fractions. In our studies, extract and fractions were active against tested fungal strains and methanol extract of *R. equisetiformis* leaves may be used in future to cure diseases caused by bacteria and fungus strains used in present research. Some of the organic compounds detected in the methanol extract and fractions have steroids, tannins, saponins, flavonoids, alkaloids, anthraquinone. These compounds have been reported to exhibit antimicrobial activity by Field and Lettinga (1992) and Scalbert (1991). The phytochemicals may have antimicrobial property through different mechanisms. As per literature review, it was observed that Verastegui et al. (2007) examined the antimicrobial studies and showed that the extracts tested were active against the fungus strains. Tannins have been found to form the irreversible complexes with proline rich protein resulting in the inhibition of protein synthesis in microbes (Parekh and Chanda, 2007). These observations therefore support the use of *R. equisetiformis* in herbal cure remedies. The presence of phytochemicals in *R. equisetiformis* supports the traditional medicinal use of this plant in the treatment of various diseases.

Haemolytic activity for cytotoxicity assay

Cytotoxicity assay was carried out by haemolytic activity against human red blood cells (RBCs). The percentage haemolysis of different extract and fractions of *R. equisetiformis* is detailed in Table 4. The highest percentage of haemolytic activity was observed in absolute methanol extract (4.96) and the lowest observed in aqueous fraction (1.89). The haemolytic activity value found in the fractions was: aqueous (1.89), *n*-butanol (4.02), chloroform (2.96), ethyl acetate (2.65), *n*-hexane (2.12) and absolute methanol (4.96).

Table 4. Cytotoxicity assay by haemolytic activity as a percentage of haemolysis caused by *R. equisetiformis* extract and fractions.

Samples	Percentage (%) of RBCs lysis
Aqueous	1.89 ± 0.020^{g}
n-butanol	4.02 ± 0.040^{c}
Chloroform	2.96 ± 0.015^{d}
Ethyl acetate	2.65 ± 0.02^{e}
n-hexane	2.12 ± 0.015^{f}
Methanol	4.96 ± 0.040^{b}
Phosphate buffer saline (PBS)	Not detected^{h}
Triton X-100	99.39 ± 1.06^{a}

The values are the average of triplicate samples (n = 3) ± S.D., ($p < 0.05$); the superscript letters represent significant differences.

The Triton X-100 was used as positive control. The mechanical stability of the erythrocytic membrane is a better sign of the effect of various *in vitro* studies by chemical constituents for the screening of cytotoxicity. It is known that in the infectious diseases haemolysis occurred due to the action of the microbes (Sharma and Sharma, 2001). So in the present study, the effect of plant extract and fractions for haemolytic activity was evaluated. The percentage lysis of human erythrocytes was below 5.0%, so it can be concluded that the extract and fractions may have minor cytotoxicity.

Conclusion

R. equisetiformis extract and fractions showed significant antioxidant potential in terms of scavenging free radicals. The DPPH scavenging and linoleic acid oxidation assays showed the antioxidant potential of the plant. The methanol extract by H_2O_2 induced oxidative damage in plasmid pBR322 DNA was evaluated and it was found that it protected the DNA, which may be due to the presence of phytochemicals which showed antioxidant properties. The potential of the extract and fractions against selected bacterial and fungal strains showed antimicrobial properties. The cytotoxicity of the plant extract and fractions were assayed by haemolytic activity against human RBCs and the percentage lysis studied *in vitro*. It was observed that the plant may have a minor cytotoxicity as the percentage lysis of RBCs was found to be below 5.0%. Due to the minor cytotoxicity the plant may be used as a herbal medicine.

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