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Screening of *Ranunculus sceleratus* for enzyme inhibition, antibacterial and antioxidant activities

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Abstract

Enzyme inhibition potential of various fractions of *Ranunculus sceleratus* was checked against *a*-glucosidase, butyrylcholinesterase, acetylcholinesterase and lipoxygenase enzymes. *n*-Butanol fraction showed very good activity (77.5 ± 1.0% inhibition at 0.1 mg/mL) against α-glucosidase. Its IC_{50} value was 35.7 ± 1.0 µg/mL comparable to quercetin (IC_{50} value 16.5 ± 0.4 µg/mL). Antibacterial activity was checked against five bacterial strains by 96-wells microplate assay using ciprofloxacin, a standard antibiotic. Chloroform, ethyl acetate, *n*-butanol and aqueous fractions showed excellent activity against *Pseudomonas aeruginosa*, (MIC at 7.1, 7.8, 5.6 and 5.3 respectively), which is greater than standard antibiotic ciprofloxacin (MIC 10.0). The antioxidant potential of all the fractions was evaluated. Ethyl acetate soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 80.9 ± 1.2% inhibition of DPPH radical at a concentration of 30 µg/mL. These results suggest that *R. sceleratus* is a valuable herb, which inhibits the oxidative stress mechanism that lead to degenerative diseases.

Introduction

Medicinal plants have always had an important place in the therapeutic armory of mankind. *Ranunculus sceleratus* Linn. is an annual or perennial herbaceous plant, which is often found on damp terrain, riversides, and small water bodies. This species originated in the northern hemisphere and it is widely distributed in China. *R. sceleratus* biosynthesizes and releases functional chemicals including ranunculin, protoanemonin and anemonin. It is widely used in traditional Chinese medicine (Wu et al., 1999) having excellent therapeutic effects (Mei et al., 2012). The fresh or dry plant can be used to treat cancer of the esophagus and the breast (Li, 1999). In addition to its medicinal value, *R. sceleratus* has other potential applications. Recent studies suggest that it is capable of purifying organic sewage and the industrial wastewater containing abundance of heavy metals. *R. sceleratus* has also been considered as a potential bio-indicator of eutrophication in aquatic habitats (Xu et al., 2004).

Alzheimer's disease is a chronic neurological disorder characterized by memory impairment, behavioral disturbances, and deficits in activities of daily living (Herbert et al., 1995). Although the basic reason of Alzheimer's disease is not clear so far, it is firmly associated with impairment in cholinergic transmission. One of the most promising approaches for treating this disease is to enhance the acetylcholine level in brain using acetylcholinesterase (AChE) inhibitors (Enz et al., 1993).

Medicinal importance compelled us to have completed biological screening of *R. sceleratus* with the aim of searching new drugs. In the present work, we described the *in vitro* enzyme inhibition, antibacterial and antioxidant activities of *n*-hexane, ethyl acetate, chloroform, *n*-butanol soluble fractions and aqueous fraction of *R.*

sceleratus, comparatively, by different standard methods, to introduce new drug candidates for the treatment of Alzheimer's and other diseases.

Materials and Methods

Collection, identification and extraction

The plant *R. sceleratus* was collected from vicinity of district Lahore, Punjab, Pakistan in December 2012, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen (GC. Herb. Bot. 673) has been deposited in the herbarium of the same university. The shade-dried ground whole plant (15 kg) was exhaustively extracted with methanol (2.5 L × 4) at room temperature. The extract was concentrated under vacuum at low temperature (35°C) using rotary evaporator. A crude extract (184 g) was obtained, which was dissolved in distilled water (1 L) and partitioned with *n*-hexane (1 L × 3), chloroform (1 L × 3), ethyl acetate (1 L × 3) and *n*-butanol (1 L × 3) respectively. These organic fractions and remaining water fraction was concentrated separately on rotary evaporator to yield *n*-hexane soluble fraction (28 g), chloroform soluble fraction (35 g), ethyl acetate soluble fraction (40 g), *n*-butanol soluble fraction (20 g) and remaining aqueous fraction (40 g) respectively. The residues thus obtained were used to evaluate their *in vitro* enzyme inhibition, antioxidant and antibacterial activities.

In vitro enzyme inhibition assays

R. sceleratus was screened for *in vitro* inhibition of four enzymes i.e. *a*-glucosidase, butyrylcholinesterase, acetylcholinesterase and lipoxygenase by following methods:

a-Glucosidase assay

The *a*-glucosidase inhibition activity was performed by modifying the spectrophotometric method developed by Pierre et al., (1978). Total volume of the reaction mixture of 100 µL contained 70 µL of 50 mM phosphate buffer saline, pH 6.8, 10 µL (0.5 mM) test compound, followed by the addition of 10 µL (0.057 units) enzyme. The contents were mixed, pre incubated for 10 min at 37°C and pre-read at 400 nm. The reaction was initiated by the addition of 10 µL of 0.5 mM subs-trate (*p*-nitrophenyl glucopyranoside). Quercetin was used as positive control. After 30 min of incubation at 37°C, absorbance was measured at 400 nm using Synergy HT microplate reader. All experiments were carried out in triplicates. The percent inhibition was calculated by the following equation,

%Inhibition = Control - Test/Control × 100

IC_{50} values (concentration at which there is 50% in enzyme catalyzed reaction) compounds were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

Cholinesterase inhibition assays

Butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) inhibition activities were measured spectrophotometrically according to standard method (Ellman et al., 1978) with slight modifications. Total volume of the reaction mixture was 100 µL containing 60 µL, Na_2H PO_4 buffer, 50 mM and pH 7.7. Ten microliter of test compound 0.5 mM/well, followed by the addition of 10 µL (0.5 unit/well) BChE and AChE separately. The contents were mixed and pre-read at 405 nm and then pre-incubated for 10 min at 37°C. The reaction was initiated by the addition of 10 µL of 0.5 mM/well substrate (butyrylthiocholine bromide and acetylthiocholine iodide separately) followed by the addition of 10 µL DTNB, 0.5 mM/well. After 30 min of incubation at 37°C, absorbance was measured at 405 nm. Synergy HT (BioTek, USA) 96-well plate reader was used in all experiments. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM/well) was used as positive control. The percent inhibition was calculated by the help of following equation:

%Inhibition = (1-Abs of test compound/Abs of control) x 100

IC_{50} values (concentration at which there is 50% enzyme inhibition) of compounds were calculated using EZ–Fit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA).

Lipoxygenase assay

Lipoxygenase (LOX) activity was assayed according to the reported method (Baylac et al., 2003) but with slight modifications. A total volume of 200 µL assay mixture contained 140 µL sodium phosphate buffer (100 mM, pH 8.0), 20 µL test compound and 15 µL (600U) purified lipoxygenase enzyme (Sigma, USA). The contents were mixed and pre-read at 234 nm and pre incubated for 10 min at 25°C. The reaction was initiated by addition of 25 µL substrate solution. The change in absorbance was observed after 6 min at 234 nm. Synergy HT (BioTek, USA) 96-well plate reader was used in all experiments. All reactions were performed in triplicates. The positive and negative controls were included in the assay. Baicalein (0.5 mM/well) was used as a positive control. The percentage inhibition was calculated by formula given below:

%Inhibition = (1-Abs of test compound/Abs of control) x 100

IC_{50} values (concentration at which there is 50% enzyme inhibition) of compounds were calculated using EZ–Fit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA).

Antibacterial assay

Strains used

The samples were individually tested against a set of microorganisms, including two Gram-positive bacteria: *Staphylococcus aureus*, API Staph TAC 6736152, *Bacillus subtilis* PCSIR-B-248, three Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Salmo-nella typhae* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 27853. The pure bacterial strains were obtained from Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. Purity and identity were verified by the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37ºC in Nutrient agar (Oxoid).

Antibacterial screening

The antibacterial activity was performed in sterile 96-wells microplates under aseptic environments. The method is based on the principle that microbial cell number increases as the microbial growth proceeds in a log phase of growth which results in increased absorbance of broth medium (Kaspady et al., 2009). Three Gram-negative and two Gram-positive bacteria were included in the study. The organisms were maintained on stock culture agar medium. The test samples with suitable solvents and dilutions were pipetted into wells (20 µg/well). Overnight maintained fresh bacterial culture after suitable dilution with fresh nutrient broth was poured into wells (180 µL). The initial absorbance of the culture was strictly maintained between 0.12-0.19 at 540 nm. The total volume in each well was kept to 200 µL. The incubation was done at 37ºC for 16-24 hours with lid on the microplate. The absorbance was measured at 540 nm using microplate reader, before and after incubation and the difference was noted as an index of bacterial growth. The percent inhibition was calculated using the formula:

Inhibition (%) = 100 *(X – Y) /X

Where X is absorbance in control with bacterial culture and Y is absorbance in test sample. Results are mean of triplicate (n = 3; ± SEM). Ciprofloxacin was taken as standard. Minimum inhibitory concentration (MIC) was measured with suitable dilutions (5-30 µg/well) and results were calculated using EZ-Fit5 Perrella Scientific Inc. Amherst USA software, and data expressed as MIC_{50}.

Antioxidant activity

Antioxidant activity of *R. sceleratus* was checked by five different methods.

Ferric reducing antioxidant power (FRAP) assay

The reducing capacity of herbal extracts was calculated according to the method of Benzie and Strain (1996) with some modifications. The solutions of plant samples and that of Trolox were prepared in methanol (500 µg/mL). The herb samples were allowed to react with FRAP solution in the dark for 30 min. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The FRAP values were determined as micromoles of trolox equivalents per mL of sample by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE µM/m.

DPPH radical scavenging activity

The DPPH radical scavenging effect of various fractions of herb was determined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the method of Lee and Shibamoto (2001). Absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples was calculated according to the formula:

Antiradical activity = $A_{control}$ - A_{sample} / $A_{control}$ × 100

Ferric thiocyanate (FTC) assay

The antioxidant activities of various fractions of herb on inhibition of linoleic acid peroxidation were assayed by ferric thiocyanate method (Valentao et al., 2002). The antioxidant activity was expressed as percentage inhibition of peroxidation (IP %):

[IP% = {1- (abs. of sample) / (abs. of control)} × 100].

The antioxidant activity of BHT was assayed for comparison as reference standard.

Total antioxidant activity

The total antioxidant activities of various fractions of plant were evaluated by phospho-molybdenum complex formation method (Prieto et al., 1999). The absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT). All determinations were assayed in triplicate and mean values were calculated.

Total phenolic contents

Total phenolics of various fractions of plant were determined by the method of Makkar et al., (1993). Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. Results were expressed in GAE / mg/g.

Statistical analysis

All the experiments were performed three times (n = 3) and the data was subjected to one way analysis of

variance (ANOVA) followed by post-hoc Tukey's test. Statistical analysis was performed by statistical software. All the data were expressed as ± S.E.M. Differences at p=0.05 were considered statistically significant.

Results and Discussion

The current study was undertaken to screen medicinal plant *R. sceleratus* for *in vitro* inhibition of four enzymes i.e. *a*-glucosidase, butyrylcholinesterase, acetylcholinesterase and lipoxygenase (Table I). The major function of *a*-glucosidase is to hydrolyze the 1,4 glycosidic link-age from the non-reducing end of the α-glucosides, *a*-linked oligosaccharide, and *a*-glucans substrates to produce *a*-D-glucose (Chiba et al., 1997). *a*-Glucosidase inhibitors are molecules or compounds that are used as oral antidiabetic drugs for patients with type-2 diabetic mellitus. The inhibitors of enzyme can retard the liberation of D-glucose of oligosaccharides and disaccharides from dietary complex carbohydrates and delay glucose absorption, resulting in reduced hyperglycemia. Therefore, inhibition of α-glucosidase is considered important in managing type-2 diabetes.

Acetyl and butyryl cholinesterases are responsible for the termination of acetylcholine at cholinergic synapses. The major function of AChE is to catalyze the hydrolysis of the neurotransmitter acetylcholine and termination of the nerve impulse in cholinergic synapses (Quinn et al., 1987). It has been found that BChE is present in significantly higher quantities in Alzheimer's plaques than in the normal age related non dementia of brains. Hence, the search for new cholinesterase inhibitors is considered to be an important and ongoing strategy to introduce new drug candidates for the treatment of Alzheimer's disease and other related diseases. Cholinesterase inhibitors increase the amount of acetylcholine available for neuronal and neuromuscular transmission through their ability to reversibly or irreversibly. A variety of neurological and neuromuscular disorders involve a diminution of cholinergic activity. Often the most effective treatments are ligands which inhibit the breakdown of acetylcholine. Lipoxygenases catalyze the addition of molecular oxygen to fatty acids containing a cis-1, 4-pentadiene system to give unsaturated fatty acid hydro peroxide (Clapp et al., 1985). It has been found that these lipoxygenase products play a key role in variety of disorders such as bronchial asthma, inflammation and tumor angiogenesis.

It was observed from the results (Table I) that *n*-butanol fraction possessed very good activity against α-glucosidase, as compared with quercetin, a reference standard drug. It showed 77.5 ± 1.0% inhibition of enzyme at concentration of 0.1 mg/mL. Its IC_{50} value was calculated as 35.7 ± 1.0 µg/mL as compared to quercetin which showed IC_{50} value 16.5 ± 0.4 µg/mL. Aqueous and ethyl acetate fraction also showed good activity having % inhibition values 32.1 ± 0.6 and 26.2 ± 0.6 respectively. *n*-Hexane fraction showed moderate activity against α-glucosidase having %inhibition value 19.2 ± 0.3. Chloroform fraction showed moderate activity against butyryl cholinesterase having IC_{50} value 31.4 ± 1.0 µg/mL. None of the fractions showed activity against acetyl cholinesterase. Ethyl acetate and *n*-butanol fractions showed good activity against lipoxygenase having IC_{50} values 36.4 ± 0.7 and 41.1 ± 0.7 µg/mL respectively as compared to baicalein, a reference standard, which showed IC_{50} value 22.7 ± 1.4 µg/mL.

Many low molecular weight metabolites are present in higher plants which provide them protection from the various microbial infections. A number of barriers provide disease resistance in the plants including physical appressoria, lignifications and defensive proteins. These metabolites inhibit the spore germination of microbes.

Antibacterial activity was checked against two Gram-positive bacteria i.e. *S. aureus* and *B. subtilis* and three

Table I

Enzyme inhibition activities of various fractions of *Ranunculus sceleratus* against α-glucosidase, butyrylcholines-terase, acetylcholinesterase and lipoxygenase

Sample (fraction)	α-Glucosidase activity	α-Glucosidase activity	BchE activity	BchE activity	AchE activity	AchE activity	LOX activity	LOX activity
	%Inhibition 0.1 mg/mL	IC_{50} µg/mL	%Inhibition 0.1 mg/mL	IC_{50} µg/mL	%Inhibition 0.1 mg/mL	IC_{50} µg/mL	%Inhibition 0.1 mg/mL	IC_{50} µg/mL
n-Hexane	19.2 ± 0.3	NIL	9.5 ± 0.4	NIL	15.4 ± 0.4	NIL	29.5 ± 0.5	NIL
$CHCl_3$	15.5 ± 0.5	NIL	72.8 ± 0.8	31.4 ± 1.0	5.1 ± 0.2	NIL	19.2 ± 0.4	NIL
EtOAc	26.2 ± 0.6	NIL	67.5 ± 0.8	35.4 ± 0.5	38.6 ± 0.7	NIL	72.2 ± 0.7	36.4 ± 0.7
n-BuOH	77.5 ± 0.9	35.7 ± 0.9	21.8 ± 0.7	NIL	18.4 ± 0.6	NIL	68.1 ± 0.9	41.1 ± 0.7
Aqueous	32.1 ± 0.6	NIL	11.5 ± 0.4	NIL	23.5 ± 0.6	NIL	14.1 ± 0.6	NIL
Control[a]	Quercetin[a]	16.5 ± 0.4	Eserine[a]	0.9 ± 0.0	Eserine[a]	0.0 ± 0.0	Baicalein[a]	22.7 ± 1.4

All results are presented as mean ± standard mean error of three assays. [a]Standard reference drugs

Table II

Percentage inhibition of various fractions of *R. sceleratus* against Gram positive and Gram negative bacteria

Samples	%Inhibition				
	S. typhi (+)	*E. coli* (-)	*P. aeroginosa* (-)	*B. subtilis* (+)	*S. aerus* (+)
n-Hexane fraction	25.6 ± 0.5	27.3 ± 0.0	34.3 ± 1.1	35.8 ± 0.9	26.8 ± 0.3
Chloroform soluble fraction	60.4 ± 0.6	62.7 ± 0.8	70.6 ± 1.4	62.7 ± 3.8	58.2 ± 1.2
Ethyl acetate soluble fraction	72.3 ± 2.3	62.8 ± 2.4	71.6 ± 0.5	53.9 ± 2.6	64.2 ± 0.0
n-Butanol soluble fraction	61.7 ± 0.8	64.3 ± 0.6	74.4 ± 2.1	54.2 ± 2.6	63.8 ± 2.6
Remaining aqueous fraction	61.4 ± 0.9	67.9 ± 0.3	71.7 ± 0.6	59.4 ± 0.1	64.0 ± 1.4
Ciprofloxacin[a]	90.5 ± 1.2	90.1 ± 0.4	92.3 ± 1.9	91.3 ± 2.0	93.1 ± 2.6

All results are presented as mean ± standard mean error of three assays; [a]Standard reference drug

Table III

MIC_{50} activities of various fractions of *Ranunculus sceleratus* against Gram positive and Gram negative

Samples	MIC $_{50}$				
	S. typhi (+)	*E. coli* (-)	*P. aeroginosa* (-)	*B. subtilis* (+)	*S. aerus* (+)
n-Hexane fraction	-	-	-	-	-
Chloroform soluble fraction	10.4 ± 0.4	11.6 ± 0.3	7.1 ± 0.5	15.5 ± 0.7	12.0 ± 0.8
Ethyl acetate soluble fraction	10.8 ± 0.3	10.7 ± 0.2	7.8 ± 0.8	9.1 ± 0.1	10.8 ± 0.3
n-Butanol soluble fraction	10.4 ± 0.4	10.9 ± 0.6	5.6 ± 0.3	12.1 ± 0.4	11.2 ± 0.2
Remaining aqueous fraction	11.3 ± 0.3	10.7 ± 0.5	5.3 ± 0.4	9.1 ± 0.6	± 10.5 ± 0.8
Ciprofloxacin[a]	8.1 ± 0.2	8.2 ± 0.1	10.0 ± 0.1	9.0 ± 0.0	8.1 ± 0.2

Table IV

IC_{50}, total antioxidant activity, FRAP values, total phenolics and lipid peroxidation of *Ranunculus sceleratus*

Sample	IC_{50} µg/mL				
Chloroform soluble fraction	47.0 ± 1.0	0.8 ± 0.0	158.0 ± 1.1	73.3 ± 1.0	21.6 ± 0.9
Ethyl acetate soluble fraction	85.0 ± 0.6	1.0 ± 0.0	238.5 ± 1.1	97.1 ± 1.0	53.7 ± 1.6
n-Butanol soluble fraction	44.1 ± 0.8	1.0 ± 0.0	148.0 ± 0.9	79.6 ± 1.0	48.0 ± 1.5
Remaining aqueous fraction	81.5 ± 1.3	0.6 ± 0.0	46.0 ± 1.3	64.6 ± 0.5	18.2 ± 0.5
Ascorbic acid[a]	58.9 ± 1.8	-	-	-	-
BHT[a]	-	0.8 ± 1.2	-	-	62.5 ± 1.1
Blank	-	-	20.4	16.5	-

All results are presented as mean ± standard mean error of three assays. [a]Standard antioxidant

Gram-negative bacteria i.e. *E. coli*, *S. typhae* and *Pseudomonas aeruginosa* by 96-well microplate assay using ciprofloxacin, a standard antibiotic, as positive control. Percentage inhibitions and MIC_{50} were measured. The results have been summarized in Table II and III respectively. It was observed that *n*-hexane soluble fraction showed very less activity. Chloroform fraction showed good activity against *P. aeroginosa* (%inhibition: 70.6 and MIC at 7.1 respectively), moderate activity against *B. subtilis* and *E. coli* (%inhibition: 62.7; 62.7 and MIC at 15.5 and 11.6 respectively). Ethyl acetate fraction showed good activity against *S. typhae* and *P. aeruginosa* (%inhibition: 72.3, 71.6 and MIC at 10.8 and 7.8 respectively). *n*-Butanol and aqueous fractions showed very good activity against *S. typhae* and *P. aeruginosa* (MIC at 5.6 and 5.3 respectively).

The results mentioned as good were found significant ($p<0.05$). The good antibacterial activity of ethyl acetate fraction was attributed to the presence of different flavonoids.

The FRAP assay measures the reducing ability of antioxidants (Shahid et al., 2013). This assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ) forming an intense blue Fe^{2+}–TPTZ complex with an absorbance maximum at 593 nm. Increasing absorbance indicates an increase in reductive ability. The FRAP values of the studied fractions were calculated and it was found that among all the fractions the ethyl acetate soluble fraction

Table V

DPPH radical scavenging activity of *Ranunculus sceleratus*

Sample (fraction)	Conc. (µg/mL)	Scavenging of DPPH radical(%) ± S.E.M[a]
n-Hexane	1000	84.6 ± 1.7
	500	59.9 ± 1.2
	250	38.5 ± 1.6
	120	30.0 ± 1.0
Chloroform	500	77.9 ± 1.1
	250	55.4 ± 1.2
	120	30.8 ± 1.1
Ethyl acetate	30	80.9 ± 1.2
	15	60.7 ± 1.3
	8	49.1 ± 0.6
n-Butanol	60	80.8 ± 1.1
	30	61.7 ± 1.3
	15	40.8 ± 1.3
Remaining aqueous	1000	77.1 ± 1.1
	500	59.2 ± 1.3
	250	47.2 ± 0.9
	120	31.3 ± 1.4
	60	20.0 ± 1.0
Ascorbic acid[b]	125	79.4 ± 1.7
	60	59.1 ± 1.6
	30	30.1 ± 0.6

[a]Standard mean error of three assays; [b]a reference standard anti-oxidant

showed highest FRAP value (238.5 ± 1.1 TE µM). FRAP values exhibited by *n*-butanol soluble fraction and chloroform soluble fraction were 148 ± 0.9 TE µM and 158.5 ± 1.1 TE µM respectively while that of aqueous fraction and *n*-hexane fraction were found to be poor (Table IV). High FRAP values obtained for more polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule reduction of DPPH radical was observed by the decrease in absorbance at 517 nm where as colour changes from purple to yellow. The various fractions of *R. sceleratus* significantly reduced DPPH radicals.

It was found (Table V) that activity increases by increasing the concentration of the fractions in the assay. The various concentrations of ethyl acetate soluble fraction exhibited highest percent of inhibition of DPPH radical as compared to other fractions. It showed 80.9 ± 1.2% inhibition of DPPH radical at a concentration of 30 µg/mL. The various concentrations of the fractions which showed percent inhibition greater than 50% were found to be significant ($p<0.05$) when compared with negative control i.e. blank. IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds. IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process. A lower value would reflect greater antioxidant activity of the fraction (Ebrahimzadeh et al., 2008). The IC_{50} values of the studied fractions were calculated (Table IV). Ethyl acetate soluble fraction exhibited lowest IC_{50} value i.e. 44.1 ± 0.8 µg/mL as compared to other studied fractions, relative to ascorbic acid, a standard reference anti-oxidant, having IC_{50} value 58.9 ± 1.8. Chloroform fraction also showed good IC_{50} value (47.0 ± 1.0 µg/mL), while *n*-butanol soluble fraction showed moderate value (85.0 ± 0.6 µg/mL). Very poor IC_{50} values were found for *n*-hexane soluble fraction and remaining aqueous fraction.

The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities (Huda-Faujan et al., 2009). The FTC assay measures the amount of peroxide value in the beginning of the lipid peroxidation, where ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion will then unite with ammonium thiocyanate producing ferric thiocyanate, a red-colored substance. The darker the color, the higher will be the absorbance. The inhibition of lipid peroxidation was checked for all the fractions. The results (Table IV) showed that ethyl acetate soluble fraction showed highest percent inhibition of lipid peroxidation i.e. 53.7 ± 1.6%. *n*-butanol fraction also exhibited good value (48.0 ± 1.5%) while *n*-hexane soluble fraction (11.0 ± 0.1%), chloroform soluble fraction (21.6 ± 0.9%) and remaining aqueous fraction (18.2 ± 0.5%) didn't show good results. The results were compared with BHT having percent inhibition 62.5 ± 1.1%.

The total antioxidant activity of the studied fractions was measured spectrophotometrically by phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of phosphate/Mo (V) compounds with a maximum absorption at 695 nm (Shahid et al., 2013). From results (Table IV), it was observed that ethyl acetate soluble fraction showed highest total antioxidant activity i.e. 1.0 ± 0.0 as compared to other fractions. *n*-Butanol fraction also showed good total antioxidant activity (1.0 ± 0.0). Chloroform soluble fraction showed moderate activity (0.8 ± 0.0) while *n*-hexane soluble fraction (0.6 ± 0.1) and remaining aqueous fraction (0.6 ± 0.0) didn't show good activity. The results were compared with butylated hydroxyltoluene (BHT), a reference standard whose total antioxidant activity was found to be 0.8 ± 1.2.

Phenolic compounds and flavonoids have been reported to be associated with anti-oxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Valentao et al., 2002). Table IV

shows the phenolic concentration in the different fractions, expressed as milligram of gallic acid equivalents (GAEs) per gram of fraction. Among these five fractions ethyl acetate soluble fraction possessed the highest amount of total phenolics compounds i.e. 97.1 ± 1.0 GAE /mg/g) followed by the *n*-butanol soluble fraction (79.6 ± 1.0 GAE/mg/g), chloroform soluble fraction (73.3 ± 1.0 GAE mg/g), remaining aqueous fraction (64.6 ± 0.5 GAE/mg/g), *n*-hexane soluble fraction exhibited the lowest total phenolic content (23.5 ± 1.5 GAE/mg/g) respectively.

Conclusion

The chloroform, ethyl acetate, *n*-butanol and aqueous fractions of *R. sceleratus* have potent enzyme inhibition, antimicrobial and antioxidant effects.

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Conflict of Interest

Authors declare no conflict of interest

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