In Vitro Assessment of Antibacterial, Enzyme Inhibition and Oxidative Stress Activities of Various Fractions of Ranunculus sceleratus

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Abstract

The aims of the present investigation were to assess the enzyme inhibition, antibacterial and antioxidant activities of various fractions of. *Ranunculus sceleratus* Linn. The methanolic extract of plant was partitioned sequentially with solvents of increasing polarity. Enzyme inhibition studies were done against four enzymes i.e. *α*-glucosidase, butyrylcholinesterase, acetylcholinesterase and lipoxygenase. It was observed from the results that *n*-butanol fraction possessed very good activity (77.49±0.94 % inhibition at 0.1 mg/ml) against α-glucosidase, as compared with quercetin, a reference standard drug. Its IC_{50} value was calculated as 35.69±0.93 µg/ml as compared to quercetin (IC_{50} value 16.47±0.39 µg/ml). Antibacterial activity was checked against five bacterial strains by 96-wells microplate assay using ciprofloxacin, a standard antibiotic, as positive control. Chloroform, ethyl acetate, *n*-butanol and aqueous fractions showed excellent activity against *Pseudomonas aeruginosa*, (MIC at 7.09, 7.82, 5.62 and 5.26 respectively) which is greater then standard antibiotic ciprofloxacin (MIC 10.03). Ethyl acetate *n*-butanol and aqueous fraction also showed good activity against *Escherichia coli* (MIC at 10.56, 10.88 and 10.74 respectively). The antioxidant potential of all the fractions was evaluated by different methods. The results revealed that ethyl acetate soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 80.9±1.23% inhibition of DPPH radical at a concentration of 30 µg/ml. The IC_{50} value of this fraction was 44.07±0.76 µg/ml, relative to ascorbic acid, having IC_{50} 58.90± 1.76 µg/ml. It also showed highest total antioxidant activity (1.04±0.03) as well as highest FRAP value (238.5±1.12 TE µM/ml), highest total phenolic contents (97.08±1.03 GAE mg/g) and highest value of inhibition of lipid peroxidation (53.70±1.59%) as compared to the other studied fractions. The results obtained from this study suggest that *Ranunculus sceleratus* is a valuable herb, which due to the presence of larger quantities of enzyme inhibition, antibacterial and antioxidant compounds inhibits the oxidative stress mechanism that lead to degenerative diseases.

Key words:

Ranunculus sceleratus Linn.; Antimicrobial potential; Antioxidant potential; *α*-glucosidase, Lipoxygenase; Cholinesterase

1. Introduction

Medicinal plants have always had an important place in the therapeutic armory of mankind. Up to 80% of populations in developing countries are totally dependent on plants for their primary health care. *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).
Many studies have confirmed the role of *R. sceleratus* as a traditional Chinese medicine, especially as an antibiotic, antiphlogostic, diarrhea cure, and mastitis remedy. It is capable of promoting blood circulation by removing blood stasis, expelling cold, relieving swelling, and removing excessive heat from the liver and the gall bladder. It can also cure internal abscess, malaria, scrofula, snake or scorpion venom, and acute icteric hepatitis (PRI, 2003). It also posses strong antifungal effects (Mei et al., 2012).

Misra and Dixit (1980) discovered that protoanemonin and anemonin extracted from *R. sceleratus* leaves had a strong antimicrobial function. The stems of *R. sceleratus* contain numerous flavonoids, which were identified by Dong et al. It is well-known that flavonoids compound, which are important secondary metabolites in plants, have many roles in fat reduction, anti-thrombotic and anti-arrhythmic therapy, and as antioxidants. Tricin is a functional component in most types of buttercups and it was detected in *R. sceleratus* (Dong et al., 2007). Tricin has a potent resistance to exterior oxidation and it can be used to treat cancer in humans.

Alzheimer's Disease (AD) is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in activities of daily living (Herbert, et al., 1995). Although the basic reason of Alzheimer's disease (AD) is not clear so far, AD 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). Several AChE inhibitors are being investigated for the treatment of Alzheimer's disease. However, only tacrine, donezepil, rivastigmine and galanthamine have been approved by the Food and Drug Administration in the United States (Zarotsky et al., 2003).
AChE inhibitors have become the remarkable alternatives in treatment of AD. However, the present drugs (donepezil, rivastigmin and tacrine) with AChE inhibitory activity possess some side effects and are effective only against the mild type of AD and there has been no drug available with butrylcholinesterase (BChE) inhibitory activity to present, yet (Schneider, 2001). Consequently, it is compulsory to develop new drugs in order to combat AD.
Arachidonate 5-lipoxygenase is the key enzyme in leukotriene biosynthesis and it catalyzes the initial steps in the conversion of arachidonic acid to biologically active leukotrienes. Angiogenesis, the formation of new capillary vessels from preexisting ones, underpins a number of physiological processes and participates in the development of several pathological conditions, such as arthritis and cancer (Nie and Honn, 2002). Lipoxygenases are therefore potential targets for the rational drug design and discovery of mechanism-based inhibitors for the treatment of a variety of disorders including bronchial asthma, inflammation, cancer and autoimmune diseases. Thus, search for new Lipoxygenases inhibitor seems to be a promising approach for the development of new drugs.

The development of resistant strains of bacteria has increased the need for new antibiotics. Bioassay guided fractionation of plant species may lead to the discovery of new antibacterial agents and a better understanding of how ethnomedicine can treat infections.
(Eloff, 1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria.
The history of drug discovery showed that plants are highly rich sources in the search for new active compounds and they have become a challenge to modern pharmaceutical industry. Many synthetic drugs owe their origin to plant-based complementary medicine. Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (David et al., 2004). An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Several authors demonstrated that antioxidant intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks (Anderson et al., 1998). Currently, there is growing interest towards natural antioxidants of plant resources. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems. The world is gradually turning to herbal formulations which are known to be effective against a large repertoire of diseases and ailments.

To the best of our knowledge, no work is done in detail on the *in vitro* enzyme inhibition, antibacterial and antioxidant activities of organic and aqueous fractions of *R. sceleratus*, comparative to each other, so, this 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.

2. Materials and methods

2.1. Collection, identification and extraction

The plant *Ranunculus 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 (184g) 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.

2.2. In vitro enzyme inhibition assays

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

2.2.1 α-Glucosidase assay

The *α*-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 37ºC and pre-read at 400 nm. The reaction was initiated by the addition of 10 µl of 0.5 mM substrate (*p*-nitrophenyl glucopyranoside). Quercetin was used as positive control. After 30 min of incubation at 37ºC, 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,

$$\% \text{ Inhibition} = \text{Control - Test / Control} \times 100$$

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).

2.2.2. 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 µl test compound 0.5 mM $well^{-1}$, followed by the addition of 10 µl (0.5 unit $well^{-1}$) BChE and AChE separately. The contents were mixed and pre-read at 405 nm and then pre-incubated for 10 mins at 37ºC. The reaction was initiated by the addition of 10 µl of 0.5 mM $well^{-1}$ substrate (butyrylthiocholine bromide and acetylthiocholine iodide separately) followed by the addition of 10 µl DTNB, 0.5 mM $well^{-1}$. After 30min of incubation at 37ºC, 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 $well^{-1}$) was used as positive control. The percent inhibition was calculated by the help of following equation.

$$\% \text{ Inhibition} = (1\text{-Abs of test compound/Abs of control}) \times 100$$

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).

2.2.3. 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 15µl(600U) purified lipoxygenase enzyme (Sigma, USA). The contents were mixed and pre-read at 234 nm and pre incubated for 10 minutes 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 $well^{-1}$) 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).

2.3. Antibacterial assay

2.3.1. Strains used

The samples were individually tested against a set of microorganisms, including two Gram-positive bacteria: *Staphylococcus aureus* (*S. aureus*), API Staph TAC 6736152, *Bacillus subtilis* (*B. subtilis*) PCSIR-B-248, three Gram-negative bacteria: *Escherichia coli* (*E. coli*) ATCC 25922, *Salmonella typhae* (*S. typhae*) ATCC 14028 and *Pseudomonas aeruginosa* (*P. 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 37 ºC in Nutrient agar (NA, Oxoid).

2.3.2. 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 37^{o}C 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:

$$\text{Inhibition (\%)} = 100 * (X - Y) / X$$

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 (5-30µg/ well) and results were calculated using EZ-Fit5 Perrella Scientific Inc. Amherst USA software, and data expressed as MIC_{50}.

2.4. Antioxidant activity

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

2.4.1. 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^{-1}). The herb samples were allowed to react with FRAP solution in the dark for 30 minutes. 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^{-1}.

2.4.2. 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:

$$\text{Antiradical activity} = A_{control} - A_{sample} / A_{control} \times 100$$

2.4.3. 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 %)

$$[IP\% = \{1- (\text{abs. of sample}) / (\text{abs. of control})\} \times 100].$$

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

2.4.4. 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.

2.4.5. 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^{-1}.

2.5. 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.

3. Results and discussion

3.1. Enzyme inhibitory potential

Herbal medicines have been used for treating numerous ailments since time immemorial. Pakistan has a valuable heritage of herbal remedies and like many of the developing countries its rural population relies on this due to low cost and ready availability. It was therefore considered to be of interest to determine the efficacy of a medicinal plant commonly used by traditional practitioners for their effects. The current study was undertaken to screen medicinal plant *R. sceleratus* for in vitro inhibition of four enzymes i.e. *α*-glucosidase, butyrylcholinesterase, acetylcholinesterase and lipoxygenase and the results have been summarized in the table 1. The major function of *α*-glucosidase is to hydrolyze the 1,4 glycosidic linkage from the non-reducing end of the α-glucosides, *α*-linked oligosaccharide, and *α*-glucans substrates to produce *α*-D-glucose (Chiba et al., 1997). *α*-Glucosidase inhibitors are molecules or compounds that are used as oral anti-diabetic 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.

Insert Table 1 here

It was observed from the results (Table 1) that *n*-butanol fraction possessed very good activity against α-glucosidase, as compared with quercetin, a reference standard drug. It showed 77.49±0.94 % inhibition of enzyme at concentration of 0.1 mg/ml. Its IC_{50} value was calculated as 35.69±0.93 µg/ml as compared to quercetin which showed IC_{50} value 16.47±0.39 µg/ml. Aqueous and ethyl acetate fraction also showed good activity having % inhibition values 32.11±0.57 and 26.18±0.63 respectively. *n*-hexane fraction showed moderate activity against α-glucosidase having % inhibition value 19.16±0.28. Chloroform fraction showed moderate activity against butyryl cholinesterase having IC_{50} value 31.44±0.98 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 36.44±0.73 and 41.09±0.66 µg/ml respectively as compared to baicalein, a reference standard, which showed IC_{50} value 22.69±1.41 µg/ml.

3.2. Antibacterial screening

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.

Insert Table 2 here

Antibacterial activity was checked against two gram-positive bacteria i.e. *Staphylococcus aureus* and *Bacillus subtilis* and three gram-negative bacteria i.e. *Escherichia coli*, *Salmonella typhae* and *Pseudomonas aeruginosa* by 96-well microplate assay using ciprofloxacin, a standard antibiotic, as positive control. % age inhibitions and MIC_{50} were measured. The results have been summarized in Table 2 & 3 respectively. It was observed that *n*-hexane soluble fraction showed very less activity. Chloroform fraction showed good activity against *Pseudomonas aeroginosa* (% inhibition :70.63 and MIC at 7.09 respectively), moderate activity against *Bacillus subtilis* and *Escherichia coli* ((% inhibition : 62.73 ; 62.69 and MIC at 15.51and 11.64 respectively). Ethyl acetate fraction showed good activity against *Salmonella typhae* and *Pseudomonas aeruginosa* (% inhibition :72.30, 71.59 and MIC at 10.84 and 7.82 respectively). *n*-Butanol and aqueous fractions showed very good activity against *Salmonella typhae* and *Pseudomonas aeruginosa* (MIC at 5.62 and 5.26 respectively). The results were compared with Ciprofloxacin, a reference antibacterial drug. These observations have been made on the basis of measurements of %age inhibition and MIC values.

Insert Table 3 here

Statistical analysis of variance (ANOVA) and the Duncan *t*-test supported the experimental results obtained. 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.

3.3. Antioxidant Activity

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 showed highest FRAP value (238.5±1.12 TE μM ml^{-1}). FRAP values exhibited by *n*-butanol soluble fraction and chloroform soluble fraction were 148±0.91 TE μM mL^{-1} and 158.5±1.09 TE μM ml^{-1} respectively while that of aqueous fraction and *n*-hexane fraction were found to be poor (Table 4). High FRAP values obtained for more polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents.

Insert Table 4 here

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic moleculeReduction 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.

Insert Table 5 here

It was found (Table 5) 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±1.23 % inhibition of DPPH radical at a concentration of 30 μg ml^{-1}. 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 4). *Ethyl acetate* soluble fraction exhibited lowest IC_{50} value i.e. 44.07±0.76μg ml^{-1} as compared to other studied fractions, relative to ascorbic acid, a standard reference antioxidant, having IC_{50} value 58.9±1.76. Chloroform fraction also showed good IC_{50} value (47.04±1.02 μg ml^{-1}), while *n*-butanol soluble fraction showed moderate value (85.05±0.58 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-coloured substance. The darker the colour, the higher will be the absorbance. The inhibition of lipid peroxidation was checked for all the fractions. The results (Table 4) showed that Ethyl acetate soluble fraction showed highest percent inhibition of lipid peroxidation i.e. 53.70±1.59 %. *n*-butanol fraction also exhibited good value (47.98±1.48 %) while *n*-hexane soluble fraction (11.02±0.13 %), chloroform soluble fraction (21.57±0.92 %) and remaining aqueous fraction (18.24±0.54 %) didn't show good results. The results were compared with BHT having percent inhibition 62.48±1.07 %.

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 4), it was observed that *ethyl acetate* soluble fraction showed highest total antioxidant activity i.e. 1.04±0.03 as compared to other fractions. *n*-butanol fraction also showed good total antioxidant activity (0.99±0.02). Chloroform soluble fraction showed moderate activity (0.81±0.03) while *n*-hexane soluble fraction (0.62±0.08) and remaining aqueous fraction (0.60±0.02) didn't show good activity. The results were compared with butylated hydroxytoluene (BHT), a reference standard whose total antioxidant activity was found to be 0.85±1.22.

Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Valentao et al., 2002). Table 4 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. 97.08±1.03 GAE / mg g^{-1}) followed by the *n*-butanol soluble fraction (79.58±0.98 GAE / mg g^{-1}), chloroform soluble fraction (73.34±1.04 GAE mg g^{-1}), remaining aqueous fraction (64.58±0.54 GAE / mg g^{-1}), *n*-hexane soluble fraction exhibited the lowest total phenolic content (23.5±1.545 GAE / mg g^{-1}) respectively.

4. Conclusion

This study concluded that chloroform, ethyl acetate, *n*-butanol and aqueous fraction of plant have potent enzyme inhibition, antimicrobial and antioxidant effects. It was observed from the results (Table 1) that *n*-butanol fraction possessed very good activity against α-glucosidase, as compared with quercetin, a reference standard drug. It showed 77.49±0.94 % inhibition of enzyme at concentration of 0.1 mg/ml. Its IC_{50} value was calculated as 35.69±0.93 µg/ml as compared to quercetin which showed IC_{50} value 16.47±0.39 µg/ml. Chloroform fraction showed moderate activity against butyryl cholinesterase having IC_{50} value 31.44±0.98 µg/ml. Ethyl acetate and *n*-butanol fractions showed good activity against lipoxygenase having IC_{50} values 36.44±0.73 and 41.09±0.66 µg/ml respectively as compared to baicalein, a reference standard, which showed IC_{50} value 22.69±1.41 µg/ml. Antibacterial activity was checked against various bacterial strains and the ethyl acetate fraction showed good activity against *Salmonella typhae* and *Pseudomonas aeruginosa* (% inhibition :72.30, 71.59 and MIC at 10.84 and 7.82 respectively). *n*-Butanol and aqueous fractions also showed very good activity against *Salmonella typhae* (MIC at 10.40 and

11.28 respectively) and *Pseudomonas aeruginosa* (MIC at 5.62 and 5.26 respectively). The results were compared with Ciprofloxacin, a reference antibacterial drug. Our results of antioxidant assays justified and supported the popular usage of *Ranunculus* species as traditional remedies for some infections. It was observed from the results that ethyl acetate soluble fraction and *n*-butanol soluble fraction showed good antioxidant activity. It showed 80.9±1.23 % inhibition of DPPH radical at a concentration of 30 µg ml^{-1}. The IC_{50} of this fraction was 44.07±0.76 µg ml^{-1}, relative to ascorbic acid, having IC_{50} 58.94±1.76 µg ml^{-1}. It also showed highest total antioxidant activity (1.01±0.08) as well as highest FRAP value (238.5±1.12 TE / µM ml^{-1}), highest total phenolic contents (97.08±1.03 GAE / mg g^{-1}) and highest value of inhibition of lipid peroxidation (53.70±1.54%) as compared to the other studied fractions. So, these fractions are potentially valuable sources of natural antimicrobials, enzyme inhibitors and bioactive materials and can be used in pharmacological preparations to produce safe, potent and non-toxic drugs.

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Table 1 Enzyme inhibition activities of various fractions of *Ranunculus sceleratus against* α-glucosidase, butyrylcholinesterase, acetylcholinesterase and lipoxygenase.

Samples	α-Glucosidase activity		BchE activity		AchE activity		LOX activity	
	%Inhibition 0.1mg/ml	IC_{50} µg/ml	%Inhibition 0.1mg/ml	IC_{50} µg/ml	%Inhibition 0.1mg/ml	IC_{50} µg/ml	%Inhibition 0.1mg/ml	IC_{50} µg/ml
n-Hexane fraction	19.16±0.28	NIL	9.49±0.39	NIL	15.37±0.44	NIL	29.48±0.53	NIL
$CHCl_3$ fraction	15.54±0.48	NIL	72.81±0.79	31.44±0.98	5.12±0.19	NIL	19.22±0.40	NIL
EtOAc fraction	26.18±0.63	NIL	67.47±0.81	35.39±0.47	38.63±0.71	NIL	72.15±0.68	36.44±0.73
n-BuOH fraction	77.49±0.94	35.69±0.93	21.81±0.74	NIL	18.44±0.57	NIL	68.07±0.85	41.09±0.66
Aqueous fraction	32.11±0.57	NIL	11.50±0.43	NIL	23.46±0.59	NIL	14.09±0.64	NIL
Control[a]	Quercetin[a]	16.47±0.39	Eserine[a]	0.86±0.002	Eserine[a]	0.04±0.001	Baicalein[a]	22.69±1.41

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

Table 2 %age Inhibition activities of various fractions of *Ranunculus sceleratus* against *Gram +Ve* and *Gram –Ve* bacteria.

Samples	%age Inhibition				
	Salmonella typhi (+)	*Escherichia coli* (-)	*Pseudomonas aeroginosa* (-)	*Bacillis subtilis* (+)	*Staphylococcus aerus*(+)
n-Hexane fraction	25.65±0.50	27.34±0.00	34.29±1.07	35.85±0.86	26.81±0.35
$CHCl_3$ fraction	60.41±0.61	62.69±0.81	70.63±1.44	62.73±3.83	58.22±1.17
EtOAc fraction	72.30±2.30	62.80±2.43	71.59±0.48	53.94±2.59	64.20±0.00
n-BuOH fraction	61.71±0.81	64.25±0.65	74.41±2.13	54.15±2.58	63.85±2.58
Aqueous fraction	61.41±0.95	67.93±0.32	71.71±0.59	59.39±0.05	63.97±1.41
Ciprofloxacin[a]	90.54±1.18	90.13±0.43	92.31±1.87	91.29±2.1	93.11±2.6

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

Table 3 MIC_{50} activities of various fractions of *Ranunculus sceleratus* against *Gram +Ve* and *Gram –Ve* bacteria.

Samples	MIC $_{50}$				
	Salmonella typhi (+)	*Escherichia coli* (-)	*Pseudomonas aeroginosa* (-)	*Bacillis subtilis* (+)	*Staphylococcus aerus*(+)
n-Hexane fraction	-	-	-	-	-
$CHCl_3$ fraction	10.41±0.45	11.64±0.31	7.09±0.49	15.51±0.75	11.99±0.76
EtOAc fraction	10.84±0.34	10.56±0.24	7.82±0.78	9.14±0.13	10.77±0.32
n-BuOH fraction	10.40±0.44	10.88±0.56	5.62±0.32	12.15±0.44	11.20±0.25
Aqueous fraction	11.28±0.32	10.74±0.51	5.26±0.45	9.14±0.56	±10.52±0.77
Ciprofloxacin[a]	8.12 ± 0.21	8.22 ± 0.12	10.03 ± 0.1	8.96 ± 0.02	8.12 ± 0.21

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

Table 4 IC_{50}, total antioxidant activity, FRAP values, total phenolics and lipid peroxidation of different fractions of *Ranunculus sceleratus*

Sample	IC_{50} µg ml^{-1}	Total antioxidant activity	FRAP value TE µM / ml^{-1}	Total phenolics *GAE* / mg g^{-1}	Inhibition of Lipid Peroxidation %
n-Hexane soluble fraction	57.75±1.85	0.62±0.08	34±1.45	23.5±1.45	11.02±0.13
Chloroform soluble fraction	47.04±1.02	0.818±0.03	158±1.09	73.34±1.04	21.57±0.92
Ethyl acetate soluble fraction	85.05±0.58	1.04±0.03	238.5±1.12	97.08±1.03	53.70±1.59
n-Butanol soluble fraction	44.07±0.76	0.99±0.02	148±0.91	79.584±0.98	47.98±1.48
Remaining aqueous fraction	81.48±1.30	0.60±0.02	46±1.29	64.58±0.54	18.24±0.54
Ascorbic acid[a)]	58.9 ±1.76	-	-	-	-
BHT [a)]	-	0.85±1.22	-	-	62.48±1.07
Blank	-	-	20.37	16.49	-

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

Table 5 DPPH radical scavenging activity of *Ranunculus sceleratus*

Sample	Conc. (µg ml^{-1})	Scavenging of DPPH radical(%)±S.E.M[a)]
n-Hexane soluble fraction	1000	84.597±1.7
	500	59.9±1.2
	250	38.53±1.6
	120	29.98±0.97
Chloroform soluble fraction	500	77.819±1.08
	250	55.45±1.23
	120	30.85±1.09
Ethyl acetate soluble fraction	30	80.9±1.23
	15	60.7±1.34
	8	49.07±0.65
n-Butanol soluble fraction	60	80.79±1.09
	30	61.74±1.34
	15	40.76±1.26
Remaining aqueous fraction	1000	77.1±1.08
	500	59.19±1.3
	250	47.25±0.93
	120	31.3±1.45
	60	20±0.98
Ascorbic acid[b)]	125	79.45±1.69
	60	59.09±1.58
	30	30.11±0.65

[a)] standard mean error of three assays.
[b)] a reference standard antioxidant
*$p < 0.05$ when compared with negative control i.e. blank/solvent ($p < 0.05$ is taken as significant)