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Antioxidant and Antimicrobial Activities of an Ethnobotanically Important Plant *Holmskioldia sanguinea* Retz. of District Kotli, Azad Jammu & Kashmir

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Abstract

In the present study, the various extracts of the plant *Holmskioldia sanguinea* Retz., were studied for their antioxidant and antimicrobial activities. Antioxidant potential of these extracts was evaluated by four methods: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity, total antioxidant activity, ferric reducing antioxidant power (FRAP) assay and ferric thiocyanate assay along with the determination of their total phenolic contents. The results revealed that among these extracts the methanol and chloroform extract showed good antioxidant potential. Their IC_{50} values were found to be 18.12±1.32 and 32.52±0.12µg/ml respectively as compared to BHT, a reference standard, having IC_{50} 12.52 ± 0.89. Methanol extract showed highest total antioxidant activity i.e. 1.142±0.08. It also showed good FRAP value (92.15±1.06 TE µM/mL), highest total phenolic contents, i.e. 74.83±1.14 GAE mg/g as well as highest value of percent inhibition of lipid peroxidation, i.e. 49.13±0.37 with standard BHT, i.e. 62.93 ± 0.78. The highest zone of inhibition was formed by methanol extract, i.e. 47±1.72 mm against *E.coli*. The MIC results revealed that the methanolic extract shows more resistance against *E. coli*, i.e. 0.010 µg/ml.

Key words: Holmskioldia sanguinea Retz., DPPH assay, total antioxidant activity, FRAP value, total phenolics, Inhibition of lipid peroxidation (%), Antimicrobial, MIC.

Introduction

The free radical reactions are usually produced in the mitochondrial respiratory system. Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (El-Hela *et al.* 2010). Antioxidants are considered as possible protection agents reducing oxidative damage of human body from ROS and retard the progress of many chronic diseases as well as lipid peroxidation (Ajaib *et al.* 2011). In general, there are two basic categories of antioxidants, natural and synthetic. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Riaz *et al.* 2012). Thus, the interest in natural antioxidants has been increased considerably. As resources of natural antioxidants much attention has been paid to plants (Abbasi *et al.* 2012).

Holmskioldia sanguinea Retz. belongs to family Verbenaceae and has long been used in folkloric medicines for the treatment of various ailments. This plant is not very common and found only in Mansuh hills District Kotli Azad Jammu & Kashmir. The flowering period of this plant was October-January. Locally this plant is called as Turk's Turban. Its Leaves and bark is used as tonic and wound healer (Ajaib 2012). To the best of our knowledge no detailed work is done on the various extracts of the whole plant of *Holmskioldia sanguinea*, therefore, in the present investigation, we described the comparative *in vitro* antioxidant potential of aqueous and organic fractions of this species by four methods: 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging, total antioxidant activity by phosphomolybdenum complex (PC) method, Ferric Reducing Antioxidant Power (FRAP) assay and ferric thiocyanate assay along with determination of their total phenolic contents relative to conventionally used standards. Variety of antioxidants are present in plant extracts therefore different antioxidant assays are required to access their antioxidant potential as one or two antioxidant assays cannot evaluate all types of antioxidants present in plant extracts. Antibacterial activities were checked against two gram-positive bacteria i.e. *Streptococcus faecelis* and *Staphylococcus aureus*, and two gram-negative bacteria i.e. *Escherichia coli* and *Pseudomonas aeruginosa.* Antifungal activities were checked against two fungi i.e. *Aspergillus niger* and *Aspergillus oryzae*.

Material and Methods

Plant Material

The fresh whole plants of *Holmskioldia sanguinea* Retz. were collected from District Kotli, Azad Jammu and Kashmir in March, 2011 and identified with help of Flora of Pakistan (Jafri & Ghafoor 1974). The voucher specimen was numbered, i.e. 2225 deposited in Dr. Sultan Ahmad Herbarium, Department of Botany, GC University, Lahore.

Micro-organisms

Gram –ve, Gram +ve bacteria and fungi were obtained from King Edward Medical College University and PCSIR Laboratories Lahore, Pakistan.

Extraction and Fractionation of Antioxidants

About 250 gm shade-dried ground whole plant was extracted successively with non- polar and polar solvents, like petroleum ether, chloroform and methanol and water by maceration for 8 days in each of the solvents respectively. The extracts were concentrated on *rotary evaporator* and the residues thus obtained were used to evaluate their *in vitro* antioxidant potential as well as antibacterial and

antifungal activities.

Chemicals and Standards

DPPH (2,2-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), trolox, gallic acid, Follin Ciocalteu's phenol reagent and BHT (Butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (petroleum ether, chloroform, methanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride, ferrous chloride, hydrocloric acid, sodium acetate and acetic acid from Merck (Pvt.) Ltd. (Germany).

1. Antioxidant Assays

Following antioxidant assays were performed on all the extracts:

(a) DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of various extracts of plant was examined and compared with a standard antioxidant, butylated hydroxytoluene (BHT) using the method of Lee *et al.* (2001) and Shahzadi *et al.* (2012).

(b)Total Antioxidant Activity by Phosphomolybdenum Complex Method

The total antioxidant activity of various extracts was evaluated by phosphomolybdenum complex formation method, following Ajaib *et al.* (2011) and Prieto (1999).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out according to Benzie and Strain (1996) with some modifications.

(c) Total Phenolic Contents

Total phenolics of various extracts of plant were determined using the method of Makkar *et al.,* 1993.

(d)Ferric Thiocyanate (FTC) Assay

The antioxidant activities of various extracts of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method of Valentao *et al.* (2002).

2. Antimicrobial activity

All the crude extracts were studied for their antibacterial activity using well diffusion method according to Ajaib *et al.* (2011) and Ferreira *et al*. (1996).

Minimum Inhibitory Concentration (MIC) of only the methanolic extract was carried out according to Murray *et al.* (1999) using modified Broth dilution assay with the help of Spectrophotometer at 595nm in mg/ml.

Statistical Analysis

All the measurements were obtained in triplicate and statistical analysis was applied on Microsoft excel and the data was expressed as ± S.E.M.

Results and Discussion

DPPH radical scavenging activity

Reduction of DPPH radical was observed by the decrease in absorbance at 517 nm where as colour changes from purple to yellow. The various extracts of *Holmskioldia sanguinea* significantly reduced DPPH radical. It was observed from the results (table 1) that activity was increased by increasing the concentration of the fractions in the assay. The various concentrations of methanol extract exhibited highest percent inhibition of DPPH radical as compared to other studied extracts. It showed 71.08 ± 1.35 inhibition of DPPH radical at a concentration of 60 µg/ml. IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH radical. It 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 extract. Methanol extract exhibited lowest IC_{50} value i.e. 18.12±1.32µg/ml as compared to other studied fractions. Chloroform extract also showed good activity. It showed IC_{50} value 32.52±0.12 µg/ml.

Petroleum ether and aqueous extract showed IC_{50} values 499.54±0.30 and 120.78±0.52 µg/ml respectively. The results were expressed relative to BHT (butylated hydroxytoluene), a reference standard, having IC_{50} 12.52 ± 0.89 (table 2)

Total Antioxidant Activity by Phosphomolybdenum Complex Method

It was revealed from the results that methanol extract showed highest total antioxidant activity i.e. 1.142±0.08 as compared to other fractions. Chloroform extract also showed good activity i.e. 0.913±0.02. Petroleum ether and aqueous extractdisplayed moderate activity (0.373±0.01 and 0.523 ± 0.28 respectively). The results were compared with BHT, a reference standard having total antioxidant activity 1.293 ± 0.09 (table 2).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP values of the studied fractions were calculated and results have been shown in table 2. Among all the extracts methanol extract showed highest FRAP value (92.15±1.06 TE µM/mL). Chloroform extract also showed good FRAP value i.e. 87.66±1.45 TE µM/mL while petroleum ether and aqueous extract showed poor FRAP values. High FRAP values obtained for polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents. The value of blank was found to be 10.35.

Total Phenolic Contents

Table 2 shows the phenolic concentration in the different extracts, expressed as milligram of gallic acid equivalents (GAEs) per gram of fraction. The methanol extract showed the highest amount of total phenolic compounds (74.83±1.14 GAE mg/g) while that of chloroform extract was found to be 53.17±0.22 GAE mg/g. Petroleum ether and aqueous extract showed very less amount of total phenolic contents. The results were compared with the blank having value 9.17 ± 0.76.

Ferric Thiocyanate (FTC) Assay

Highest percentage of inhibition of lipid peroxidation was exhibited by methanol extract (49.13±0.37%). Chloroform extract displayed moderate value i.e. 37.56±0.22 while petroleum ether and aqueous extract showed poor FRAP values. The inhibition of lipid peroxidation by BHT was found to be 62.93 ± 0.78%. The results have been shown in table 2.

Antibacterial and Antifungal Activities

The results for antibacterial and antifungal activities of various extracts of the plant have been shown in table 3. It was observed that the highest zone of inhibition was formed by methanol extract i.e. 47±1.72 mm against the bacteria *E. coli*. It also showed good activity against *Streptococcus aureus* (43±0.33 mm). Aqueous extract showed good activity against *Staphylococcus faecelis* (35±0.53 mm) and *Staphylococcus aureus* (35±0.73 mm). Methanol and aqueous extract showed highest antifungal activities with zone of inhibitions 46±0.53 mm and 46±0.53 mm respectively. Methanol extract also showed good activity against *Aspergillus oryzae* i.e. 37±1.13 mm. Chloroform extract displayed good activity against both the used fungi. All the other results were found moderate or non-significant. The results were compared with the standard antibiotic drugs whose zones of inhibitions have been given in table 4.

Minimum inhibitory concentrations (MIC) were also calculated and the results have been shown in table 5. The MIC results revealed that the methanolic extract shows more resistance against *E. coli*, i.e. 0.010 µg/ml.

see Table 1.

see Table 2.

see Table 3.

see Table 4.

see Table 5.

Discussion

In the present study, the antioxidant and antimicrobial activities of *Holmskioldia sanguinea* were tested to verify ethnobotanical knowledge. It was noticed that all the plant extracts had shown different antioxidant potential through DPPH assay. The significant IC_{50} values (concentration of sample required to scavenge 50% free radical), were found to be 18.12±1.32 and 32.52±0.12µg/ml respectively as compared to BHT, a reference standard, having IC_{50} 12.52 ± 0.89. DPPH radical scavenging activity is widely used to evaluate antioxidant activities in a relatively short time. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The effects of phenolic compounds on DPPH radical scavenging are thought to be due to their hydrogen donating ability (Miladi *et al* 2008). It is reported that the decrease in the absorbance of DPPH radical caused by phenolic compound is due to the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation and is visualized as a discoloration from purple to yellow (Benzie and Strain 1996). DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples. This method is based on the reaction of DPPH radical that is characterized as a stable free radical with deep violet colour and any substance that can donate hydrogen atom to DPPH thus reduces it to become stable diamagnetic molecule Abbasi *et al.* 2010).

In phosphomolybednum complex method, the reduction of Mo (VI) to Mo (V) took place by various fractions of plant which was detected at 695nm by spectrophotometer due to the formation of green phosphate Mo (V) compounds (Makkar *et al.* 1993 & Valentao *et al.* 2002). A higher absorbance indicates a higher antioxidative activity.

FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. 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.

It has been suggested that the phenolic content of plant materials is correlated with their antioxidant activity. The phenolic compounds may contribute directly to antioxidative action. The antioxidative activities observed can be explained both the different mechanisms exerted by different phenolic compounds and to the synergistic effects of different compounds. The antioxidants have different functional properties, such as reactive oxygen species scavenging e.g. quercetin and catechin, inhibition of the generation of free radicals and chain-breaking activity, e.g. *p*-coumaric acids and metal chelation. These compounds are normally phenolic compounds, which are effective proton donors, and include tocopherols, flavonoids and other organic acid might be these different compounds present in various extracts of *Holmskioldia sanguinea*.

Oxygen reacts with unsaturated double bond on the lipid which results in generation of free radicals and lipid hydroperoxides. Peroxidation of lipids occurs both *in vivo* and *in vitro* and gives rise to cytotoxic and reactive products. These products disturb the normal functioning of the cell and can give rise to damaged or modified DNA. Hydrogen donating antioxidants can react with lipid peroxyl radicals and break the cycle of generation of new radicals. The Ferric Thiocyanate Assay is used to measure the amount of peroxide at the beginning of lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate, a reddish pigment (Abbasi *et al.* 2011).

Antimicrobial potential of plant extracts was investigated by calculating the inhibition zones produced by plant extracts against bacterial and fungal strains. The crude extracts of *Holmskioldia*

sanguinea in different polar and non-polar solvents restricted the growth of various bacterial and fungal strains. The standard discs were used against microorganisms to make a comparison between the zones of inhibition produced by the commercially available discs and plant extracts against four different bacterial strains (Gram-positive bacteria, i. e. *Staphylococcus aureus, Streptococcus faecelis* and Gram-negative bacteria, i.e. *Escherichia coli,* and *Pseudomonas aeruginosa*). The highest zone of inhibition was formed by methanol extract, i.e. 47±1.72mm against *E.coli*. Two antifungal standard discs were used against the fungal strains, i.e. *Aspergillus oryzae* and *Aspergillus niger*. The highest zone of inhibition was formed by aqueous and methanol extracts, i.e. 46±0.53 and 46±1.4 respectively against *Aspergillus niger*.

These zones produced by the aqueous extracts might be because of the polar compound extracted in aqueous medium being readily soluble in it, like tannins, terpenoids and alkaloids. Cheruiyot *et al.,* (2009) reported similar results while determining the antimicrobial activities of methanol plant extracts of *Psidium guajava* leaves against *P. aeruginosa, E. coli and S. aureus*. Similarly Ramzi *et al*., (2005) investigated the antimicrobial activity of many plants, including *Boswellia elongata* etc. against *S. aureus* and found the methanolic extracts showing the highest activity. This might be because of many phytochemical compounds including terpenoids, flavenoids, polyphenolic compounds as well as tannins expected to be extracted in methanol. The MIC (Minimum Inhibitory Concentration) results revealed that the methanolic extract showed more resistance against *Pseudomonas aeruginosa*, i.e. 0.009 µg/ml. These results are very much similar to Saxena *et al*., (1994) while testing different concentrations of *Rhus glaba* extracts on both, Gram-negative and Gram-positive bacteria.

Conclusion

The results revealed that among the studied extracts of *Holmskioldia sanguinea* the methanol and chloroform extract showed good antioxidant potential. Their IC_{50} values were found to be 18.12±1.32 and 32.52±0.12µg/ml respectively as compared to BHT, a reference standard, having IC_{50} 12.52 ± 0.89. Methanol extract showed highest total antioxidant activity i.e. 1.142±0.08. It also showed good FRAP value (92.15±1.06 TE µM/mL), highest total phenolic contents, i.e. 74.83±1.14 GAE mg/g as well as highest value of percent inhibition of lipid peroxidation, i.e. 49.13±0.37 with standard BHT, i.e. 62.93 ± 0.78. The highest zone of inhibition was formed by methanol extract, i.e. 47±1.72mm against *E.coli*. The MIC results revealed that the methanolic extract shows more resistance against *E. coli*, i.e. 0.010 µg/ml. So it was concluded that methanol and chloroform extracts of this plant can be used for drug development as antioxidant and antimicrobial agents.

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Sr. No.	Sample	Concentration (µg/ml)	% scavenging of DPPH ± S.E.M[a)]
1.	Petroleum ether extract	1000 500 250 130	60.84 ± 1.25 50.01 ± 1.48 46.69 ± 0.98 40.06 ± 0.87
2.	Chloroform extract	250 130 60 30	69.58 ± 1.76 64.16 ± 1.05 52.41 ± 0.94 47.59 ± 0.75
3.	Methanol extract	60 30 15 8	71.08 ± 1.35 60.20 ± 1.63 51.01 ± 0.83 40.05 ± 0.58
4.	Aqueous extract	250 130 60	65.03± 1.59 54.22 ± 0.99 40.01 ± 0.67
5.	BHT[b)]	60 30 15 8	92.46 ± 0.25 74.57 ±0.39 49.61 ± 0.55 28.33 ± 0.83

Table 1. 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging activity of the various extracts of *Holmskioldia sanguinea* Retz.

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

Sr. No.	Plant Sample	IC_{50} of DPPH assay (µg/mL) ± S.E.M [a]	Total antioxidant activity± S.E.M [a]	FRAP value (TE µg/ml) ± S.E.M [a]	Total phenolics (GAE mg/g) ± S.E.M [a]	Inhibition of lipid peroxidation (%) ± S.E.M [a]
1	Petroleum ether extract	499.54±0.30	0.373±0.01	11.66±1.20	10.05±0.10	7.66±1.02
2	Chloroform extract	32.52±0.12	0.913±0.02	87.66±1.45	53.17±0.22	37.56±0.22
3	Methanol extract	18.12±1.32	1.142±0.08	92.15±1.06	74.83±1.14	49.13±0.37
4	Aqueous extract	120.78±0.52	0.527±0.03	14.33±0.36	28.66±0.45	18.71±0.34
6	BHT[b]	12.52 ± 0.89	1.293 ± 0.09	-	-	62.93 ± 0.78

Table 2: IC_{50}, total phenolics, total antioxidant activity, FRAP values and lipid peroxidation inhibition values of different extracts of *Holmskioldia sanguinea* Retz.

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

Bacteria	(mm)			
	Petroleum ether extract	Chloroform extract	Methanol extract	Aqueous extract
i) Gram-Positive Bacteria				
Streptococcus faecelis	11±0.41	8±1.45	10±1.63	35±0.53
Staphylococcus aureus	11±2.03	9±1.32	43±0.33	35±0.73
ii) Gram-Negative Bacteria				
Escherichia coli	12±1.1	10±1.00	47±1.72	20±1.17
Pseudomonas aeruginosa	0±0	0±0	0±0	22±1.73
Fungi				
Aspergillus niger	20±1.34	35±1.23	46±0.53	46±1.4
Aspergillus oryzae	0±0	30±1.57	37±1.13	17±1.0

Table 3: Zone of inhibition produced by bark and leaves extracts of *Holmskioldia sanguinea* Retz. against bacteria and Fungi

Micro-organisms	Standard disc (30µg)	Zone of inhibition (mm)
Pseudomonas aeruginosa	Amikacin	25.00±5.00
Escherichia coli	Sulfamethoxazole	9.67±0.57
Streptococcus faecelis	Ampicillin	29.67±0.57
Staphylococcus aureus	Ampicillin	19.67±0.57
Aspergillus oryzae	Nystatin	29.00±1.00
Rhizopus oryzae	Kanamycine	48.5±1.52
Aspergillus niger	Tezole	22.00±0.01

Table 4. Zone of inhibition produced by standard discs against bacteria and fungi.

Concentration of methanol extract(µL)	MIC value (µg/ml)			
	Staphylococcus aureus	*Enterococcus facaelis*	*Escheria coli*	*Pseudomonas aeruginosa*
0.1	0.112	0.061	0.051	0.261
0.2	0.011	0.284	0.057	0.117
0.3	0.547	1.121	0.345	0.094
0.4	1.149	2.382	1.126	1.164
0.5	2.467	2.446	1.259	1.865
0.6	2.426	2.335	2.444	1.053
0.7	2.245	2.221	2.281	0.030
0.8	2.198	2.210	2.124	1.012
0.9	0.024	0.030	0.019	0.359
1.0	0.013	0.016	0.010	1.336

Table 5: MIC Values of *Holmskioldia sanguinea* Retz.