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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF AN ETHNOBOTANICALLY IMPORTANT PLANT *NOTHOLIRION THOMSONIANUM* FROM DISTRICT KOTLI, AZAD JAMMU & KASHMIR

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ABSTRACT

The petroleum ether, chloroform, methanol and aqueous extracts of *Notholirion thomsonianum* (D. Don) Stapf, obtained by maceration method were tested for their antioxidant potential, antibacterial and antifungal activities. Antioxidant activity was checked by four methods: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity, total antioxidant activity, ferric reducing antioxidant power (FRAP) assay and ferric thiocyanate (FTC) assay along with the determination of their total phenolic contents. The results revealed that among these fractions the methanol soluble fraction showed highest DPPH radical scavenging activity, i.e. 91.26±0.44% inhibition of DPPH radical at a concentration of 500 µg/ml with IC_{50} value 22.08±0.69 relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.52 ± 0.89 µg/ml. Chloroform extract showed highest total antioxidant activity, i.e. 0.730±10.56 as well as highest FRAP value, i.e. 22.66±1.33 TE µM/ml of aqueous extract. Methanol, chloroform and petroleum ether extracts showed considerable amounts of total phenolic contents, i.e. 147±0.59 and 145±0.85 and 141.83±2.36 GAE mg/g respectively. Methanol extract showed good value of inhibition of lipid peroxidation, i.e. 47.19±1.25. Antimicrobial activity was evaluated by well diffusion method against some bacteria and fungi. The highest zone of inhibition was formed by aqueous extract, i.e. 35±1.31 against *E. coli* followed 32±0.32 and 32±0.9 of aqueous extract against *Streptococcus faecelis* and *Staphylococcus aureus* respectively. The highest antifungal zone of inhibition was formed by aqueous and petroleum ether extract, i.e. 40±1.1 and 37±1.73 against *Aspergillus oryzae* and *Aspergillus niger* respectively. The Minimum Inhibitory Concentration (MIC) results revealed that the methanolic extract 0.009 µg/ml showed more resistance against *Pseudomonas aeruginosa*.

Key words: *Notholirion thomsonianum*, DPPH assay, total antioxidant activity, FRAP value, Inhibition of lipid peroxidation (%), antimicrobial, MIC.

INTRODUCTION

Plants have been a source of medicine for thousands of years, and phytochemicals continue to play an essential role in medicine (Aggarwal, 2003). Medicinal plants are in greater demand due to their increased popularity, thus it is being suggested by a large number of conservation groups, that wild medicinal plants should be brought into cultivation. Numerous medicinal plants, as well as, their purified components have shown beneficial therapeutic potentials. Various herbs and other plant species are reported to show antioxidant activity. Majority of the antioxidant potential is due to the presence of flavones, flavonoids, isoflavones, anthocyanin, lignans, coumarin, catechins and isocatechins (Aqil *et al.,* 2004). Antioxidant-based drug products are being used for the treatment and prevention of complicated diseases like atherosclerosis, diabetes, stroke, Alzheimer's disease, and cancer (Devasagayam, 2004). In living organisms, free radicals are produced as a result of the normal metabolic process, and also free radical chain reactions normally occur as respiratory chain reaction in the mitochondria, through xanthene oxidase activity, liver mixed function oxidases, atmospheric pollutants and from the transitional metal catalysts, xenobiotics, and drugs. In addition to this, chemical mobilization of the fat stores in different conditions such as lactation, fever, exercise, infection, and even fasting, may result in enhanced radical activity, and damage. Oxidative injury or free radicals now appear as if the fundamental mechanism, causing a number of the human neurologic and many other disorders. Peroxidation of lipids can be initiated by the oxygen free radical, which in turn stimulates the glycation of protein, inactivation of some enzymes, and alteration in the function and structure of collagen basement and a few other membranes, and also play a role in chronic complication of diabetes (Ara and Nur, 2009).

Notholirion thomsonianum (D. Don) Stapf belonging to a monocotyledonous family Liliaceae is locally used to treat various diseases. It is a herb upto 35 cm in height. The plant is common in grassy fields of Brooth near Khuiratta and flowered during March-April. Locally this plant is called as Sanp Buti and Hazara Lily. Its leaves and bulbs are used for digestive complaints and tumors. Leaves are also bandage on wounds as anodyne

(Ajaib, 2013). No detailed work has been carried out on the various effects of extracts of the whole plant of *N. thomsonianum*. Therefore, present investigation was designed to test the comparative *in vitro* antioxidant and antimicrobial potential of aqueous and organic fractions of this species using 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. Antibacterial activity was 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 activity was checked against two fungi, i.e. *Aspergillus niger* and *Aspergillus oryzae*.

MATERIAL AND METHODS

Plant Material: The fresh whole plants of *N. thomsonianum* were collected from District Kotli, Azad Jammu and Kashmir in March, 2011 and identified with help of Flora of Pakistan Ali (2007). The voucher specimen was numbered, i.e. 2224 deposited in Dr. Sultan Ahmad Herbarium, Department of Botany, GC University, Lahore.

Micro-organisms: Gram –ve (*Escherichia coli* and *Pseudomonas aeruginosa*), Gram +ve (*Streptococcus faecelis* and *Staphylococcus aureus*) bacteria and fungi (*Aspergillus niger* and *Aspergillus oryzae)* 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 prepare plant fractions of various concentrations to evaluate their *in vitro* antioxidant potential as well as antibacterial and antifungal activities.

The solvents applied for maceration were selected according to their polarity indices from non-polar to polar as follows:

Solvent used	Polarity index (P)
Petroleum ether	0.1
Chloroform	4.1
Methanol	5.1
Water	10.2

The plant fractions were prepared in µg/ml concentrations such that for the preparation of 60 µg/ml, 0.06 g of the plant extract was transferred to separate one liter conical flask and the ultimate volume was brought to one liter with the solvent in which the extract was macerated. Likewise, the other concentrations were prepared and stored at 20˚C.

The physical analysis was conducted on the plant extracts of *N. thomsonianum* including its color, texture, order and % yield before subjecting them to further evaluations. The % extraction yield was calculated by employing the following formula:

$$\%\ \text{Extraction yield} = \frac{\text{Weight of plant extract}}{\text{Weight of initial plant sample}} \times 100$$

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).

Antioxidant Assays: Following antioxidant assays were performed on all the extracts:

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).

Total Antioxidant Activity by Phosphomolybdenum Complex Method: The total antioxidant activity of various extracts was evaluated by phosphomolybdenum complex formation method, following Prieto *et al.* (1999) and Ajaib *et al.* (2011).

Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP assay was carried out according to Benzie and Strain (1996) with some modifications.

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

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).

Antimicrobial activity: All the crude extracts were studied for their antibacterial activity using well diffusion method according to Ajaib *et al.* (2011), Ortega and Julian (1996) 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 595 nm in mg/ml.

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

RESULTS

The procedure adopted for the extraction of the components is considered to be an important factor in the determination of the commercial feasibility, yield and quality of the extract obtained. The maceration technique is considered due its cost effectiveness and good solute yield in comparison to other techniques.

The highest yield thus obtained from the bark of the *N. thomsonianum* was (15.1%). The % extraction yield of leaf of *N. thomsonianum* was 8.76%. The lowest yield obtained from the leaves of *N. thomsonianum* was 1 to 10%. The petroleum ether extract yielded the solute in the range of 1 to 3.5%. The chloroform extract yielded the solute in the range of 1 to 10% and a yield of 3 to 15.5% was obtained from the alcoholic extract of plant material. In addition, the aqueous extracts yielded 5 to 9% solute (Fig.1).

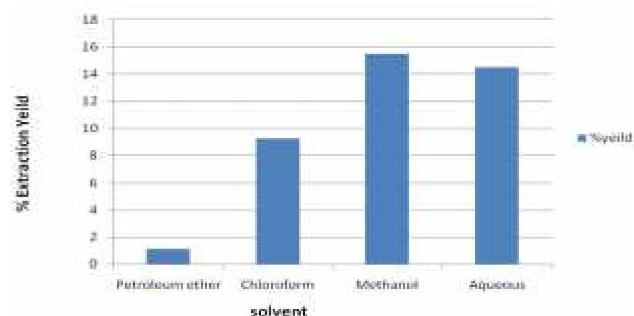

Fig. 1: Percent Extraction yield of *N. thomsonianum* whole plant.

DPPH radical scavenging activity: The various extracts of *N. thomsonianum* were tested and the values of percent scavenging of DPPH radical were recorded shown in Table 1. It was observed that activity was increased by increasing the concentration of the fractions in the assay. The various concentrations of methanol extract exhibited highest DPPH radical scavenging activity as compared to other fractions. It showed 91.26±0.44% inhibition of DPPH radical at a concentration of 500 µg/ml. The IC_{50} values were also calculated and recorded in Table 2. Lowest the IC_{50} value, greater was the DPPH radical scavenging activity. It was observed from the results that methanol extract showed lowest IC_{50} value, i.e. 22.08±0.69 relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.52 ± 0.89 µg/ml.

Total Antioxidant Activity by Phosphomolybdenum Complex Method: The total antioxidant activity of the studied samples was measured and compared with the standard antioxidant BHT (Table 2). It was revealed that chloroform extract showed highest total antioxidant activity, i.e. 0.730±10.56.

Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP values of the studied extracts were calculated, as shown in Table 2. Among all the extracts, the highest FRAP value, i.e. 22.66±1.33 TE µM/ml was found in aqueous extract methanol extract was observed.

Total Phenolic Contents: Table-2 shows the phenolic concentration in the studied fractions in milligrams of gallic acid equivalents (GAEs) per gram of fraction. Among them methanol, chloroform and petroleum ether extracts showed considerable amounts of total phenolic contents, i.e. 147±0.59, 145±0.85 and 141.83±2.36 GAE mg/g respectively.

Ferric Thiocyanate (FTC) Assay: The various extracts of plant were tested by this assay and results were tabulated in Table 2. It was observed that that methanol extract showed value of inhibition of lipid peroxidation, i.e. 47.19±1.25.

Antibacterial and Antifungal Activities: The negative control was established by evaluating the solvents in which the extracts were prepared against the bacterial and fungal strains used as test organisms (Table 3 and 4). The standard drugs were employed against all pathogenic test organisms and Zone of Inhibitions produced by different drugs were recorded (Fig. 2A and Table 5).The results for antibacterial and antifungal activities of various extracts in Table 6 showed that the highest zone of inhibition was formed by aqueous extract i.e. 35±1.31 mm against the bacteria *E. coli* (Fig. 2B, Table 6) whereas Zone of Inhibition, i.e. 10±1.65 was produced by chloroform extract against *P. aeruginosa* (Fig. 2C, Table 6). Aqueous extract showed highest antifungal activity with 40±1.1 mm Zone of Inhibition against the *Aspergillus oryzae* (Table 6) whereas petroleum ether extract also showed good activity, i.e. 37±1.73 mm against *Aspergillus niger* (Fig. 2D, Table 6). All the other results were found moderate or non-significant (Table 6). The results were compared with the standard antibiotic drugs (Fig. 2A)

whose Zones of Inhibitions have been given in Table 5. MIC revealed that the methanolic extract showed more resistance against *Pseudomonas aeruginosa*, i.e. 0.009 µg/ml at a concentration of 0.1 µL followed by 0.025 µg/ml at o.5 µL, against *Streptococcus facaelis*.

Table 1. Free radical scavenging activity of various fractions of *N. thomsonianum* using 2, 2-Diphenyl-2-picrylhydrazyl radical (DPPH).

Sr. No.	Plant Extracts of *N. thomsonianum*	Concentration in assay (µg/ml)	% scavenging of DPPH ±S.E.M[a)]
1.	Chloroform	500	60.24±0.03
		250	40.06±0.03
		130	35.78±0.05
2.	Methanol	500	91.26±0.44
		250	72.3±0.34
		130	61.75±0.22
		60	45.48±1.22
3.	Petroleum Ether	1000	34.63±2.03
		500	30.42±0.11
		250	25.30±0.21
4.	Aqueous	1000	70.48±0.37
		500	57.83±0.90
		250	46.99±0.93

Standard means and errors of three assays.

Table 2. IC_{50}, total phenolics, total antioxidant activity, FRAP values and lipid peroxidation inhibition values of *N. thomsonianum*.

Sr. No.	Plant Extracts of *N. thomsonianum*	IC_{50} of DPPH assay (µg/mL) ± S.E.M	Total antioxidant activity by Phosphomolybdenum Method (abs. at 593 nm) ± S.E.M	FRAP value TE (µg/ml) ± S.E.M	Total phenolics (GAE mg/g of sample) ± S.E.M	Inhibition of lipid peroxidation (%) ± S.E.M
1	Petroleum ether	Not detected	0.67±0.58	20±0.65	145±0.85	5.22±3.85
2	Chloroform	363.09±2033	0.730±10.56	11±0.54	141.83±2.36	16.34±3.25
3	Methanol	22.08±0.69	0.434±3068	11±1.56	147±0.59	47.19±1.25
4	Aqueous	311.33±0.64	0.221±10.56	22.66±1.33	116.67±4.85	14.43±1.34
6	BHT	12.52 ± 0.89	0.891 ± 0.13	-	-	62.93 ± 0.78

Table 3. Zone of Inhibition (mm) of solvents against the bacterial strains as negative control.

Solvents	Quantity (ml)	Zone of inhibition (mm)			
		S.aureus	*S. faecelis*	***P.aeruginosa***	***E. coli***
Petroleum ether	1.5	0±0	0±0	0±0	0±0
Chloroform	1.5	0±0	0±0	0±0	0±0
Methanol	1.5	0±0	0±0	0±0	0±0
Aqueous	1.5	0±0	0±0	0±0	0±0

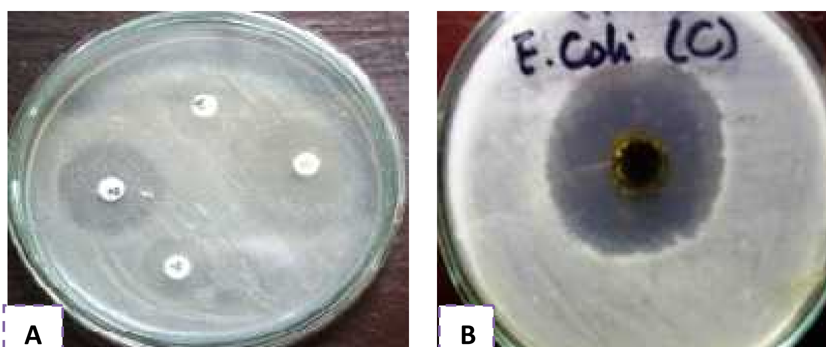

Fig. 2. Zones of Inhibition of standard drugs and extracts against different pathogenic microbes.
A. Zone of inhibition produced by standard drugs. **B.** Zone of Inhibition of aqueous extract against *E. coli*. **C.** Zone of Inhibition of chloroform extract against *P. aeruginosa*. **D.** Zone of Inhibition of Methanol extract against *A. niger.*

Table 4. Zone of Inhibition (mm) of solvents against the fungal strains as negative control.

Solvents	Quantity (ml)	Zone of inhibition (mm)	
		A. oryzae	*A. niger*
Petroleum ether	1.5	0±0	0±0
Chloroform	1.5	0±0	0±0
Methanol	1.5	0±0	0±0
Aqueous	1.5	0±0	0±0

Table 5. Zone of inhibition produced by standard discs against bacterial and fungal test strains.

Micro-organisms	Standard disc (30µg)	Zone of inhibition (mm)
Pseudomonas aeruginosa	Amikacin	23.00±4.00
Escherichia coli	Sulfamethoxazole	11.67±0.67
Streptococcus faecelis	Ampicillin	27.67±0.28
Staphylococcus aureus	Ampicillin	18.57±0.20
Aspergillus oryzae	Nystatin	30.00±1.20
Rhizopus oryzae	Kanamycin	49.6±2.05
Aspergillus niger	Tezole	25.20±1.04

Table 6. Zone of inhibition produced by whole plant extracts of *N. thomsonianum* against bacteria.

Pathogenic organisms	Zone of inhibition (mm)			
	Petroleum ether (extracts)	Chloroform (extracts)	Methanol (extracts)	Aqueous (extracts)
i) Gram-Positive Bacteria				
Streptococcus faecelis	16±0.3	17±0.43	20±0.11	32±0.32
Staphylococcus aureus	17±1.20	17±1.21	17.5±1.11	32±0.9
ii) Gram-Negative Bacteria				
Escherichia coli	22±1.77	7±2.03	32±0.42	35±1.31
Pseudomonas aeruginosa	0±1.54	10±1.65	20±1.75	25±0.98
Fungi				
Aspergillus niger	37±1.73	25±1.73	22±1.73	20±1.3
Aspergillus oryzae	25±1.73	22±2.31	0±1.21	40±1.1

DISCUSSION

In the present study, the antioxidant and antimicrobial activities of *N. thomsonianum* 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), i.e. 22.08±0.69 was observed in methanolic extract as compared to that of standard antioxidant BHT having the value of 12.52.±0.89. It was also observed that increasing the concentration of plant extract, antioxidant potential also became significant. Hence, these plant samples were found good as antioxidants and comparable with the standard, BHT. Zheng and Wang (2001) reported the antioxidant compounds from plants had been reported as active oxygen scavengers or free radicals. A considerable interest has been increased to explore natural resources having antioxidant potential and can replace synthetic antioxidants. The phosphomolybdenum complex method usually exposes antioxidants such as ascorbic acid, carotenoids, some phenolics and tocopherols. The chloroform extract had 0.730±10.56 total antioxidant activity.

Antimicrobial potential of plant extracts was observed by calculating the inhibition zones produced by plant extracts against bacterial and fungal strains. The crude extracts of *N. thomsonianum* 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*). 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 extract, i.e. 35±1.31 against the bacteria *E. coli* and 40±1.1 against the aqueous extract of fungi *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, where as Ajaib *et al.* (2013a and 2013b) also reported the similar results while working with the antimicrobial and antioxidant activities of *Rivina humilis* L., *Echinochloa colona* (Linn.) Link, and *Sporobolus coromandelianus* (Retz.) Kunth. 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: From significant antioxidant and antimicrobial results it was concluded that chloroform extract, methanol extract and aqueous extract are rich in strong bioactive constituents which would be expected to increase shelf life of foods and fortify against peroxidative damage in living systems in relation to aging, carcinogenesis and chronic diseases.

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