Biological Activities of *Opuntia Monacantha* Cladodes

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Summary: The Present study was conducted to investigate the biological activities of the plant O. *monacantha* cladodes. The plant cladodes were extracted with methanol and then fractionized with various organic solvents (*n*-hexane, chloroform, ethyl acetate and *n*-butanol). The extract and fractions thus obtained were analyzed for their phytochemical, antioxidant and antimicrobial analysis. The percentage yield of methanolic extract and various fractions was found to be 2.7-28.9%. Phytochemical analysis exposed the presence of alkaloids, tannins, saponins and flavonoids in methanolic extract. The plant extract and fractions contained total phenolic contents (14.5-249 GAE, mg/100 g of dry plant matter) and total flavonoid contents (10-433.8 CE, mg/100 g of dry plant matter). DPPH radical scavenging activity was determined by measuring IC_{50} that was found to be 41.4-838.8 µg/mL. Nevertheless, minute variation was observed in reducing power. Methanol extract exhibited highest antioxidant activity among various solvent fractions. The antimicrobial activity was determined by disc diffusion method and minimum inhibitory concentration against a panel of microorganisms. Significant variations ($p<0.05$) were observed in the results.

Key Words: *Opuntia monacantha*, anthraquinone, DPPH, flavonoids, minimum inhibitory concentration.

Introduction

Opuntia monacantha Haw. commonly known as prickly pear belongs to family Cactaceae. It is usually grown in tropical and subtropical regions [1]. It can be cultivated under restricted growth conditions that are not suitable for the growth of other fruits and vegetables [2]. The *O. monacantha* cladodes do not possess appreciable content of alkaloids and exhibit mild antitumour activity [3] The Opuntia cladodes are known to possess remarkable medicinal properties [4]. The polysaccharides isolated from the cladodes of *O. monacantha* show anti-diabetic and anti-glycated activity [5]. The antioxidant and anti-inflammatory action of *O. monacantha* exert neuroprotective effects and inhibit stomach ulceration [6].

Antioxidants are valuable compounds which protect the humans from oxidative damage caused by free radicals [7]. Medicinal plants are excellent sources of natural antioxidants which not only scavenge free radicals but also lower the lipid peroxidation in the blood or liver [8].

The need for the present research was to determine the phytochemical constituents, antioxidant and antimicrobial activities of the plant *O. monacantha* cladodes.

Results and Discussion

% yield of Methanolic Extract and Organic Fractions

The yield (g/100g) of the plant methanolic extract and various fractions for different organic solvent systems is given in Table-1. The maximum amount was extracted with methanol (28.9%), followed by *n*-hexane (6.01%), ethyl acetate (4.6%) *n*-butanol (3.4%), and chloroform (2.7%). The amount of materials that can be extracted from a plant depends on the nature and amount of solvent used mixing during extraction procedure and it is possible sample to sample variation in extracted materials [9].

Table: 1 %age yield of *O. monacantha* cladodes methanolic extract and various organic fractions.

Samples	Yield (g/100g)[a]
Methanol	**28.9±1.21**
Chloroform	**2.70±0.12**
Ethylacetate	**4.60±0.23**
***n*-butanol**	**3.40±0.47**
***n*-hexane**	**6.01±0.93**

[a]Values are mean±SD of three separate experiment

Phytochemical Analysis

Phytochemical constituents were analyzed in methanolic extract of the plant cladodes (Table-2).

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Alkaloids, flavonoids, tannins and saponins were found to be present in methanolic extract while steroids and anthraquinones were absent.

Table-2: Phytochemical constituents of *O. monacantha* cladodes Methanol extract.

Antimicrobial agents	Methanol
Alkaloids	+
Flavonoids	+
Tannins	+
Steroids	-
Saponins	+
Anthraquinones	-

Antioxidant Activity of Plant Extract and Fractions

The amount of total phenolic and total flavonoid contents (TPC and TFC) of the plant given in Table-3. The methanolic extract and different organic fractions were found to contain the TPC and TFC ranged from 14.5-249 mg/100g as GAE and 10-443.8 mg/100g as CE, respectively. TPC and TFC extracted in different solvents were found to decrease in the order: methanol > *n*-butanol > ethyl acetate > chloroform > *n*-hexane. There are many reports illustrating the effect of polarity on the extraction of TPC and TFC. More the plolarity of the solvent more TPC and TFC will be extracted. Since phenolics and flavonoids are polar compounds so they are extracted more in methanol as compared to other solvents [10-12]. Soil and growing conditions have pronounced effects on the amount of TPC which need to be extracted from the plant. The ability of a particular solvent to dissolve endogenous compounds causes the difference in the amount of TPC and TFC extracted from plant [13].

The plant methanolic extract and its different fractions exhibited different radical scavenging activity having IC_{50} value of 41.4-838.6 µg/mL. Methanolic extract exhibited lowest IC_{50} (41.4 µg/mL) followed by *n*-butanol (330.2 µg/mL), ethyl acetate (588.0 µg/mL), chloroform (615.2 µg/mL) and *n*- hexane (838.6 µg/mL) fractions (Table-3). These activities however were lower than those of the reference sample: BHT (19 µg/mL). The nature and amount of secondary constituents of the plant causes the variation in free radical scavenging ability [14]. The free radical scavenging activity depends upon the chemical composition of extract and fraction. Valente et al [15] reported the antioxidant activity of the MeOH extract and of the *n*-hexane, EtOAc and *n*-BuOH fractions of *O. monacantha* by measuring their ability to scavenge DPPH radicals. The samples were able to inhibit the activity of DPPH radicals in a dose dependent manner and their IC_{50} values (concentration in µg/mL required to achieve a 50% reduction in DPPH radicals) were 833 for OM-crude MeOH extract, 469 for OM–hexane fraction, 53.2 for OM–EtOAc fraction, and 278 for OM-*n*-BuOH fraction. This showed an activity enhancement for the fractions, specially the EtOAc and *n*-BuOH fractions, in comparison to the crude MeOH extract. These activities however were lower than those of the reference samples: quercetin (0.83) and ascorbic acid (6.7). These results are in agreement with our work where ethylacetate and *n*-butanol fractions showed good results to scavenge DPPH but activity was lower than reference sample (BHT).

Antioxidants activity was also determined by the measurement of reducing power of extracts/ fractions. In this assay, actually the colour of experimental solution changed from yellow to bluish green when ferric ions (Fe^{3+}) are converted to ferrous ions (Fe^{2+}) by gaining electrons. The intensity of the colour reflects the reducing potential of the components present in extract/fractions. So as the intensity of colour increases, absorption increases and hence, antioxidant potential also increases. The reducing potential (Fig. 1) at concentrations of 0.2-1.0 mg/mL was noted to be in the range of 0.51-0.77 nm. The maximum absorbance (0.77 nm) was recorded for methanol extract, while the minimum for *n*-hexane (0.51 nm) fraction. On comparing with standard ascorbic acid (0.9 nm) all the extract and fractions showed significantly ($p< 0.05$) low reducing potential. The reducing power of extract/fraction is related to the presence of reductones, which is assumed to reduce radical chains by donating hydrogen atom so the antioxidant potential is associated with reducing power [16].

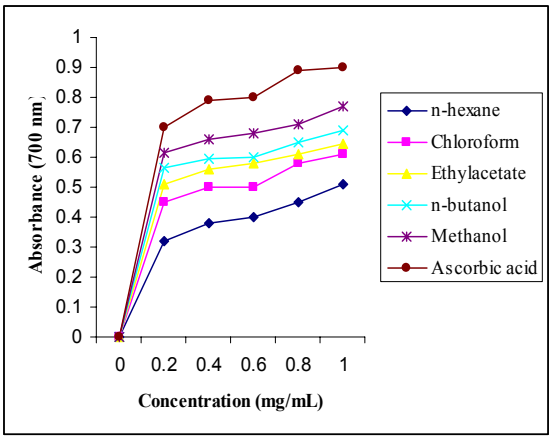

Fig. 1: Reducing potential of methanol extract and different fractions of *O. monacantha* cladodes

Antimicrobial Activity

From the results of Disc Diffusion Method (Table-4) and Minimum Inhibitory Concentration (MIC) (Table-5) it was concluded that *A. alternata* was found to be most sensitive fungus showing largest zone of inhibition (21.3 mm) and lowest MIC value (60.03 mg/mL). Of three fungal strains used, *R. solani* exhibited maximum resistance to n-hexane fraction showing no inhibition zone. No activity may be due to the fact that a particular of extract or fraction do not contain any active compound or if present its concentration is very low so as to be effective or it may have lost its activity. *A. flavus* showed maximum resistance to *n*-butanol fraction showing lowest inhibition zone (9.7 mm) and highest MIC value (283 mg/mL). The antibacterial activity was more pronounced against gram positive bacteria (*S. aureus* and *B. subtilis*) than gram negative bacteria (*E. coli* and *P. multocida*). Based on these findings, chloroform and *n*-butanol fractions showed strong activity against *S. aureus* having highest zone of inhibition (21.5 and 19 mm) and lowest MIC value (52.02 and 90.03 mg/mL). Similarly methanolic extract and chloroform fraction exhibited highest activity against *B. subtilis* showing largest zone of inhibition (19.3 and 22.8 mm) and lowest MIC value (45.15 and 70 mg/mL). The less sensitivity of gram negative bacteria than gram positive bacteria is related to their difference in cell membrane composition. The gram negative bacteria possess an outer lipopolysaccharide covering which acts as permeability barrier to the diffusion of hydrophobic compounds [17]. The differences in the antimicrobial effects may be due to the difference in phytochemical properties and differences among species. It is also possible that the active chemical constituents may not be soluble in a particular solvent.

Experimental

Plant Materials

The cladodes of *O. monacantha* were collected from botanical garden, University of Agriculture, Faisalabad, Pakistan and identified by a taxonomist, Dr. Mansoor Hameed. The specimen (Voucher No: 8009) has been deposited in the Herbarium of Botany department, University of Agriculture, Faisalabad, Pakistan.

Sample preparation

After removing the spines, fresh cladodes of the plant *O. monacantha* were washed with distilled water and then shade dried for 20 days. The grinded fine powder (865g) of cladodes was extracted with methanol (2×15 L) using an orbital shaker (Gallenkamp, UK) for 6 hours at room temperature. After filtering the extract was concentrated through rotary vacuum evaporator (Eyela, Tokyo Rikakikai Co., Ltd., Japan). This process was repeated thrice to obtain a sufficient quantity of methanol extract and stored at -4°C. The methanolic extract (250 g) was dissolved in distilled water and then fractionation was performed by using different polarity based solvents and obtained successively *n*-hexane (52 g), chloroform (24 g) ethylacetate (40 g), and *n*-butanol (30 g) fractions.

Phytochemical Analysis

Phytochemical screening of methanolic extract was carried out according to the previously reported methods [18].

Total Phenolic Contents (TPC)

TPC were determined using Folin-Ciocalteu reagent method [19]. In short, 0.5 ml of crude extract of each fraction (1 mg/mL) of plant was mixed with 0.5 mL of Folin-ciocalteu reagent (1 mL folin reagent + 4 mL distill water) and 7.5 mL deionized water and the mixture was kept for 10 min at room temperature, and then added 1.5 mL 20% sodium carbonate (w/v), mixed it thoroughly. In a water bath the mixture was heated at 40°C for 20 min. and then cooled in an ice bath; at 755 nm absorbance was taken using a spectrophotometer (U-2001, Hitachi Instrument Inc.Tokyo, Japan). Amounts of total phenolic contents were calculated by using Gallic acid calibration curve within range of 10-100 ppm (R^2 =0.9952).The results were written as Gallic acid equivalents (GAE) of dry plant matter.

Table-3: Antioxidant activities of *O. monacantha* cladodes methanol extract and different fractions[a].

Antioxidant assays	*n*-hexane	Chloroform	Ethylacetate	*n*-butanol	Methanol	BHT
[b]Total Phenolic contents	14.5±0.50	19.07±0.20	21.2±0.26	23.5±0.41	249.1±0.26	---
[c]Total Flavonoid contents	10.1±0.10	14.3±0.360	17.9±0.10	18.0±0.20	443.8±0.30	---
DPPH, IC_{50} (µg/ml)	838.6±0.10	615.2±0.20	588±0.17	330.2±0.10	41.4±0.25	19±0.17

[a]Values are mean±SD of three separate experiment
[b]Total phenolic contents calculated in mg/100g of dry plant material are expressed as gallic acid equivalent.
[c]Total flavonoid contents calculated in mg/100g of dry plant material are expressed as catechin equivalent.

Table-4: Antimicrobial potential of *O. monacantha* Cladodes by disc diffusion method.

	Microbial Strain Inhibition Zone (mm)[a] Antibiotic Methanolic extract and different organic fractions					
		n-hexane	Chloroform	Ethylacetate	*n*-butanol	Methanol
Bacteria	**Rifampcin**					
B. subtilis	**35.8±0.50**	**9.5±0.57**	**19.3±0.95**	**17.3±1.25**	**16.5±0.57**	**22.8±0.50**
P. multocida	**31.5±10**	**10.3±.50**	**12.5±1.00**	**14.3±0.95**	**15.8±0.50**	**17.8±0.500**
S. aureus	**29.8±0.50**	**17.7±0.50**	**21.5±1.00**	**17.5±0.57**	**19.0±1.15**	**18.3±0.095**
E. coli	**26.5±1.00**	**10.8±1.50**	**13.8±0.50**	**11.3±0.95**	**10.8±0.97**	**16.3±0.50**
Fungi	**Fluconazole**					
A. flavus	**22.1±1.155**	**15.8±0.50**	**14.5±0.57**	**11± 1.15**	**9.7±0.50**	**11.8±1.25**
A. alternata	**32.3±1.258**	**17.5±0.57**	**19.8±0.50**	**19.5±1.00**	**21.3±0.97**	**17±1.15**
R. solani	**20.5+1.00**	**Nill**	**13.5±1.00**	**15.3±1.50**	**17.8±0.50**	**16 ± 0.81**

Values are mean ± SD of three separate experiments
[a]Diameter of inhibition zone (mm) including disc diameter of 6 mm.

Table-5: Minimum inhibitory concentration (MIC) of *O. monacantha* Cladodes extracts and fractions against some microbial strains

	Microbial Strain MIC (mg/mL). Antibiotic Methanolic extract and different organic fractions					
		n-hexane	Chloroform	Ethylacetate	*n*-butanol	Methanol
Bacteria	**Rifampcin**					
B. subtilis	**3.04±0.00**	**213± 0.10**	**180 ± 0.00**	**145± 0.05**	**145±0.15**	**45.1±0.00**
P. multocida	**2.09±0.01**	**210± 0.01**	**70.1 ± 0.01**	**110± 0.04**	**95.0± 0.01**	**72.1±0.15**
S. aureus	**5.09±0.01**	**90.0± 0.00**	**52.0 ± 0.01**	**1.13± 0.02**	**90.0± 0.05**	**60.1±0.00**
E. coli	**9.11±0.01**	**164± 0.57**	**91.1± 0.01**	**160± 0.02**	**175± 0.02**	**153±0.01**
Fungi	**Fluconazole**					
A. flavus	**13.1±0.00**	**110±0.06**	**140±0.07**	**235± 0.02**	**283± 0.05**	**204±0.1**
A. alternata	**3.01±0.01**	**170±0.06**	**157±0.16**	**113± 0.06**	**87.0±0.02**	**190±0.00**
R. solani	**19.2±0.10**	**Nill**	**101±0.05**	**83.0±0 01**	**60.0±0.05**	**71.0±0.02**

Values are mean ± SD of three separate experiments

Total flavonoid contents (TFC)

TFC were determined by following a previously reported Spectrophotometric method [20]. Briefly 0.5 mL of the plant extract/fractions (1mg/mL) was placed in a 10 mL volumetric flask, then added distilled water 5 ml and 0.3 mL of 5% $NaNO_2$ was added to each volumetric flask initially; after 5 min., 0.6 mL of 10% $AlCl_3$ was added. After another 5 min, 2 mL of 1 M NaOH was added and volume was made up with distilled water. At 510 nm absorbance of the reaction mixture was noted by using a spectrophotometer. TFC were evaluated as catechin equivalents (g/100 g of Dry plant matter).

DPPH Radical Scavenging Assay

The 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out spectrophotometrically as described by Bozin *et al*. [21]. Stock solution was prepared by dissolving 100 mg of each extract or fraction in 100 mL of methanol. From Stock solution different concentrations were made from 0.2-1 mg/mL. After that in each concentration, added 5 mL of freshly prepared DPPH having concentration 0.025g/L. After 10 minutes, absorbance of resulting solution and blank (5 mL DPPH + 1mL methanol + 10 min) was taken at 515 nm. Three replicates were recorded for each sample. According to the following equation, the inhibitory %age of DPPH was calculated:

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

where A_{blank} is the absorbance of control (containing all reagents except the test samples), and A_{sample} is the absorbance of the test samples. IC_{50} value (mg /mL) is defined as the concentration at which the scavenging activity was 50%, caused 50% neutralization of DPPH radicals, and was measured from the plot of concentration against percentage inhibition.

Determination of Reducing Power

The reducing power of the plant extract and fractions was carried spectrophotometrically [22]. Stock solution was prepared by dissolving 100 mg of each extract or fraction in 100 mL of methanol. From Stock solution different concentrations were made from 0.2-1 mg/mL. To the above 1ml of each concentration, added 2 ml phosphate buffer and 2 mL potassium ferricyanide (1%). Incubate the mixture at 50°C for 20 minutes. Then added 2 mL of 10% trichloroacetic acid, centrifuged at 3000 rpm for 10 minutes at 5°C. The upper layer of the solution was removed. At the end added 5 mL deionised water and 1 ml $FeCl_3$. At 700 nm absorbance of the reaction mixture was taken using a spectrophotometer. All samples were analyzed in triplicate.

Antimicrobial Assay of Plant Extract and Different Fractions

Microbial Strains and Preparation of Inoculum

Antimicrobial activity of the plant was evaluated against three fungal strains (*Rhizopus solani*, *Aspergillus flavus* and *Alternaria alternata*) and four bacterial strains (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pasturella multocida*). These strains were obtained from Department of Veternary Microbiology, University of Agriculture, Faisalabad. The bacterial strains were cultured overnight at 37°C in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 28 °C using potato dextrose agar (Oxoid).

Disc Diffusion Assay

Antimicrobial activity of the above mentioned methanolic extract and of various organic fractions was determined by using the disc diffusion method [23]. The discs (6 mm diameter) were impregnated with 10 mg/mL extract/fractions (100 µL/disc) placed aseptically on the inoculated agar. Discs injected with 100 µL of respective solvents were served as negative controls; Rifampcin (100 µL/disc) (Oxoid) and Fluconazole (100 µL/disc) (Oxoid) were used as positive reference for bacteria and fungi, respectively. The petri dishes were incubated at 37 ± 0.1°C for 20-24 h and 28± 0.3°C for 40-48 h for bacteria and fungi respectively. At the end of period, the inhibition zones formed on the media were measured. The positive antimicrobial activity was read based on growth inhibition zone. Evaluation of the inhibitory properties was carried out in triplicates.

Resazurin Microtitre-Plate Assay

The minimum inhibitory concentration (MIC) of the plant extracts/fractions was evaluated by a modified resazurin microtitre-plate assay reported by Sarker *et al*. [24] with little modifications.

Conclusion

The present study showed that *O. monacantha* has good biological activities. All extract and fractions of the plant exhibited good antioxidant and antimicrobial activities but methanolic extract was more potent as antimicrobial agent and it also extracted highest amount of antioxidative compoounds. So it can be a potential source of antimicrobial and antioxidant agents However, detailed spectral analysis is required to isolate and characterize the potent compounds responsible for antioxidant and antimicrobial activity and explore their beneficial role.

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