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Phytochemical and pharmacological studies on medicinally important plant *Opuntia dillenii* collected from Pakistan

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Abstract

Keeping in view the numerous useful pharmacological activities of *Opuntia* plants, we explored in detail the secondary metabolites and metals in different parts, fatty acids composition of seed oil and a detailed anti-inflammatory study of the cladodes along with acute toxicity studies of *Opuntia dillenii* collected from Pakistan. Carbohydrates, saponins, tannins, flavonoids, terpenoids and phenols were present in both the cladodes and fruit part. Terpenes and sterols were found in the cladode part while alkaloids were only present in the fruit part. Highest quantity of sodium was found in the same quantity (394.00 ppm) cladodes and fruit while potassium was abundantly present in the fruit part (15430.00 ppm). Calcium was present in a very high quantity in the cladodes (11820.00 ppm) and fruit (16419.40 ppm). Linoleic acid, a potent cyclooxygenase-2 (COX-2) inhibitor, was with highest concentration (59.153 %) among fatty acids. The crude extract displayed significant anti-inflammatory activity (50.88 %) at 300 mg/kg body weight dose after 3h dose injection. Acute toxicity tests confirmed the safety of crude extract to a dose level of up to 1000 mg/kg body weight. The present study provides a base to the natural product chemists to carry out further studies on the isolation of the secondary metabolites from *Opuntia dillenii* and explore the bio-efficacies of the individual compounds.

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Introduction

Secondary metabolites like alkaloids, flavonoids, terpenoids, saponins and polyphenolics display a lot of useful and interesting biological activities like antimicrobial, antioxidant, cholesterol lowering, antidiabetic, anticancer, antiproliferation, anti-inflammatory, analgesic, cardiotonic, immune-stimulatory *etc*. A large population of the world is still using about 35,000 medicinal plants for curing different ailments. Due to their side effects and the resistance developed by microbes to the synthetic (allopathic) drugs, natural products once again have attracted the researchers towards them for developing new natural products based herbal drugs for the treatment of different life-threatening diseases like cancer, viral infections and other infections caused by microbes (Philip *et al*., 2009; Kohtaro *et al*., 2002; Curry *et al*., 2005; Fodili and Bommel 2003; Katz *et al*., 1992; Friswell *et al*., 2010; Peiris *et al*., 2007; Mosqueda *et al*. 2012; Galanis *et al*., 2009).

Opuntia (prickly pear) genus has its place in the family Cactaceae which is consisting of 127 genera and 1750 species. *Opuntia* are well grown wild plants in most of the parts of the world. It preferably grows in semiarid areas where the propagation of other plants is not so easy. These plants generally provide protection to agricultural fields from humans and animals and act like boundary walls. They are also used as an essential part of food in some countries like USA, Northern Africa and Italy. In general, different *Opuntia* species have many important pharmacological activities like antidiabetic (Hwang *et al*., 2017) anti-atherosclerotic and antihyperlipidemic (Choi *et al*., 2002), antiulcerogenic and antioxidant (Galati *et al*., 2003), anti-viral (Ahmad *et al*., 1996), anti-inflammatory (Park *et al*., 2012), *etc*.

A very useful data regarding different biological activities like antioxidant, hypocholesterolemic, antidiabetic, antitumor, antiproliferation and immune-stimulatory, their phytochemical constituents and their use as functional food and additive use of different *Opuntia* species has been covered up in the review by Seema Patel (Patel, 2012).

Opuntia dillenii, one of the species of genus Opuntia, is also commonly known as tuna, malrachette, prickly pear and pear bush. *Oputia ficus indica* and *Opuntia dillenii* display anti-inflammatory, cholesterol and sugar lowering and analgesic activities (Galati *et al*., 2003; Park *et al*., 2001; Perfumi and Tacconi, 1996; Ennouri *et al*., 2005).

Inflammation, a defensive response, starts when a damage occurs to a local tissue. It results from several reasons like microbial infections and toxins produced by microbes, physical or chemical injury to tissues, different polluted foreign particles, some tenacious infections like tuberculosis and ulcers, prolonged and continuous exposure to different toxic agents like silica, *etc*. Inflammation performs vital roles in the body, for example, informing the body when an area is injured, removal of the agents causing inflammation, restricting the injury to that specific injured area, removal of dead tissue, healing the injured tissue, *etc*. Different pharmacological activities are well documented to be due to different secondary metabolites (Bruneton, 1995). The present study was designed to make a thorough investigation of *Opuntia dillenii* found in Pakistan for its different phytochemicals in its different parts, its seed oil composition and its anti-inflammatory activity in the hope to help the relevant researcher's directional from the very start for their research activities on *Opuntia dillenii*.

Materials and methods

General: Reagents and solvents used were of analytical grade. Flame photometer (Jenway PFP7, UK) and atomic absorption spectrometer (Hitachi Z-8000, Japan) were used for metals analysis. Gas-Chromatograph-Mass Spectrometer (GC-MS) Model QP 2010 plus (Japan) was used for fatty acids composition determination. Standard procedures were used for secondary metabolites identification. Acute toxicity test was performed following standard procedure. Carageenan induced paw edema procedure was used for the evaluation of anti-inflammatory activity at different dose levels using Digital Plethysmometer (LE 7500 Panlab S.L.).

Collection and processing of plant material

20 kg fresh plant material was collected from Attock area of Pakistan. The plant specimen was deposited in the Herbarium of Pakistan Council of Scientific and Industrial Research Laboratories Complex, Peshawar (PES-10494). The plant was separated into cladodes (modified stem), seeds and fruit part. The fresh plant material (cladodes) was extracted with ethanol and then concentrated. The extractive value in ethanol was found to be 4.9 %. The extracted material was used further for the phytochemical investigations and anti-inflammatory activity. Seeds were removed from the fruit part for its fatty acid content determination *via* GC-MS and metals analysis through atomic absorption spectrometer. The fruit pulp without seeds, apart from its secondary metabolites' studies, was also processed for metals analysis.

Ash content and metals analysis

Ash content of the cladodes, fruit pulp and seeds was determined. The dried material (3 gm each) was burnt on a bunsen burner which was kept further in a furnace for ashing at 650 ^{o}C for 5 hours. The ashed material after cooling was transferred to a desiccator and weighed after complete cooling at room temperature. The ash content of the cladodes, fruit pulp and seeds was found to be 2, 1.8 and 2.3 % respectively. The ash of each part was digested with nitric acid separately. The contents were transferred to a 50 mL volumetric flask by diluting with dilute hydrochloric acid and making the volume up to 50 mL with the help of distilled water. The samples were run for their metals content with flame photometer for the determination of sodium, potassium and calcium and with atomic absorption spectrometer for the determination of chromium, manganese, iron, nickel, zinc, lead and cadmium.

Metals concentration (Table 1) was determined using the formula;

Metal concentration (ppm) = In$R V d_f$ / W

Where InR = Instrument reading

V = Volume made for analysis

d_f = Dilution factor

W = Weight of sample taken for analysis

Seed oil composition

Chemicals and reagents procured for the gas chromatography mass spectrometry included a 37 fatty acids standard methyl ester mixture from AccuStandard, Connecticut USA. Standard methanolic sodium hydroxide solution (0.5 N) as well as analytical grade sodium chloride were purchased from Merck (Germany). Similarly, a 10 % boron triflouride methanolic solution was procured from Fluka (Switzerland). HPLC grade methanol and *n*-hexane were acquired from Fischer Scientific (UK) and a 99.9999 % Helium gas was obtained from Pak Gas (UAE).

The seeds were separated from the fruit pulp and air dried. The dried seeds were grinded and extracted with *n*-hexane at room temperature three times. The combined hexane fractions were concentrated on rotary evaporator resulting in an 8.7 % extractive value. Fatty acids present in the seed oil were converted into their methyl esters to make them volatile while following the standard reference method (AOAC, 2000). BF_3-methanol reagent was used for this purpose. The ester solution was kept in boiling water and heated further for 30 minutes after which an already prepared saturated solution of sodium chloride added to it. Extraction was made with *n*-hexane. The filtered hexane extract was subjected to GC-MS Model QP 2010 plus (Tokyo, Japan) with an automatic sampler collector model AOC-20S and an automatic injector (AOC-20i) for fatty acids determination. The results have been tabulated in Table 2.

Secondary metabolites determination

Determination of different classes of secondary metabolites of different parts of *Opuntia dillenii* was carried out following standard procedures. These procedures can be generally summarized here. Aqueous solutions of the extracts were used for the detection of carbohydrates while treating with Molisch's, Benedict's and Fehling's reagents. A violet ring with Molisch's reagent while with Benedict's reagent an orange red precipitate and with Fehling's reagent a red precipitate confirms the presence of

carbohydrates. Formation of a reddish orange precipitate with Dragondorff's reagent and a yellow precipitate with Hager's reagent shows the presence of alkaloids. Saponins can be confirmed by froth test. A 0.5 g diluted aqueous extract giving a persistent froth, after shaking vigorously, shows the presence of saponins.

The formation of a green or bluish green precipitate with ferric chloride reagent depicts the presence of tannins. Flavonoids presence can be confirmed by the appearance of a red colour in the extract with magnesium strips after a subsequent addition of HCl (conc.). A red colour precipitate formation with 1 % aqueous HCl on boiling displays the presence of phlobatannins. Chloroform solution after treatment with 10 drops of acetic anhydride and 2 drops of conc. H_2SO_4 forms a light green ring at the interface when terpenes are present. A chloroform solution of extract gives a reddish-brown precipitate with few drops of conc. H_2SO_4 (Salkowki's test) confirming the presence of terpenoids.

In the presence of cardiac glycosides, a 5 mL extract with glacial acetic acid (2 mL) and ferric chloride solution (a drop) and 1 mL conc. H_2SO_4 (Keller Kelliani's test) gives a brown, violet and a greenish layer. The extract gives a dark pink or red colour layer with Liebermann-Buchard reagent indicating the presence of sterols. A deep blue or black colour is formed with a 5 % ferric chloride solution when phenols are there. This test in known as ferric chloride test. Results from the secondary metabolites studies have been presented in Table 3.

Animals

Albino rats (*Balb-C*, 150-200 g) of either sex were utilized for pharmacological activities. The animals were kept at room temperature (25 ^{o}C) for 12 h in light and dark in the animal house of PCSIR Labs Complex Peshawar.

2.7 Acute toxicity

Acute toxicity test for the crude cladodes extract was performed on a group of 6 albino rats for each dose level. Different doses used for this purpose were 250, 500 and 1000 mg/kg per orally. The control group was fed with 10 mL/kg of saline. Any effect in the groups was observed for first 4 hours while mortality was observed after 24 hours of dose injection.

Anti-inflammatory activity

A literature known method was followed for exploring the target activity of the cladodes crude ethanolic extract (Winter *et al*., 1962). Animals were grouped in five groups, each with six rats, for this purpose. Normal saline at a dose of 10mL/kg body weight was given to Group I, group II was treated with diclofenac sodium (10 mg/kg body weight). The groups III, IV and V were administered with 100, 200 and 300 mg/kg body weight extracts intra-peritoneally respectively. 0.1 mL of 1 % carrageenan solution was used to produce acute inflammation in the subcutaneous part of the sub planter part of its right hind paw after 30 minutes of the administration of the above test doses. Carrageenan was given to the positive and negative groups in the same way as well. Digital Plethysmometer (LE 7500 Panlab S.L.) was used for the measurement of inflammation. Measurements were made after the administration of carrageenan stepwise at regular intervals that is, 1 h, 2 h, 3 h, 4 h and 5 h. The anti-inflammatory activity was determined using the equation;

$$I / \% = (\Delta A - \Delta B / \Delta A)100$$

Where I = inhibition,
ΔA is the measure in paw volume increase of the control while ΔB is the paw volume increase of the groups administered with the test sample.

Results for the anti-inflammatory activity are highlighted in Table 4 and Figure 1.

Statistical analysis

Results for the anti-inflammatory activity were expressed as Standard Error Mean (± SEM). Graph Pad Prism Ver. 6 was used for statistical analysis while for multiple comparisons, ANOVA was trailed by post hoc Dunnett's test. Statistically, the values $p <$

0.05 and $p < 0.01$ were suggested significant.

Results and discussion

Metals analysis

Mineral and heavy metals analysis was carried out with flame photometer and atomic absorption spectrometer. Highest quantity for sodium (294.00 ppm) was found in the cladodes and fruit while potassium was abundantly present in the fruit part (15430.00 ppm) apart from its appreciable amounts in the cladodes (2758.00 ppm) and seeds (1594.00 ppm). Calcium was present in a very high quantity in the cladodes (11820.00 ppm) and fruit (16419.40 ppm) while it was also present in sufficient quantities in the seeds (2191.70 ppm). Manganese (400.00 ppm), iron (42.00 ppm) and zinc (30.40 ppm) were at their highest concentrations in the cladodes.

Other heavy metals were either not detected or found in very very low quantities (Table 1).

Table 1. Metals concentration in *Opuntia dillenii* different parts.

Plant part	Concentration, ppm										
	Na	K	Ca	Cr	Mn	Fe	Ni	Cu	Zn	Pb	Cd
O-Cl[a]	394.00	2758.00	11820.00	ND[d]	400.00	42.00	1.60	2.50	30.40	3.00	ND[d]
O-Fr[b]	394.00	15430.00	16419.40	ND[d]	20.60	14.00	2.50	5.00	17.60	3.40	ND[d]
O-Sd[c]	69.70	1594.00	2191.70	ND[d]	4.20	2.50	1.50	4.50	22.80	2.30	ND[d]

[a] = *Opuntia dillenii* cladodes; [b] = *Opuntia dillenii* fruit; [c] = *Opuntia dillenii* seeds; [d] = Not detected.

Fatty acid composition of seed oil

GC-MS analysis for the esterified fatty acids was performed using external standard method. The results are summarized in Table 2 which shows that the oil is consisting of a number of useful fatty acids both saturated and unsaturated. Linoleic acid, a potent cyclooxygeanse-2 (COX-2) inhibitor, was the most abundant fatty acid (59.153 %).

The next fatty acids with the highest concentrations were palmitic acid (10.072 %), oleic acid (8.742 %), elaidic acid (6.593 %) and stearic acid (5.124 %) while the remaining fatty acids were in low concentration (less than 1 %).

Table 2. Fatty acid composition of *Opuntia dillenii* seed oil.

ID #	Name	R. Time, min	Area, volts-min	Mass, %
1	C6:0; Hexanoic acid, methyl ester	2.949	5282	0.009
2	C8:0; Caprylic acid methyl ester	4.748	2135	0.002
3	C10:0; Capric acid methyl ester	6.488	1372	0.001
5	C12:0; Lauric acid, methyl ester	8.092	16172	0.012
6	C13:0; Tridecanoic acid, methyl ester	9.194	1108	0.001
7	C14:0; Myristic acid, methyl ester	10.190	121736	0.084
9	C15:0; Pentadecanoic acid, methyl ester	11.654	17201	0.012
11	C16:0; Palmitic acid, methyl ester	13.491	14818333	10.072
12	C16:1; Palmitoleic acid, methyl ester	13.918	196886	0.657
13	C17:0; Margaric acid, methyl ester	15.540	70282	0.051
14	C17:1; Heptadecenoic acid, methyl ester	15.980	7115	0.023
15	C18:0; Stearic acid, methyl ester	17.973	7424856	5.124
16	C18:1c; Oleic acid, methyl ester	18.394	3507605	8.742
17	C18:1n9t; Elaidic acid, methyl ester	18.579	1933169	6.593
18	C18:2c; Linoleic acid, methyl ester	19.864	26945605	59.153
20	C18:3n6; g-linolenic acid, methyl ester	20.551	9219	0.025
21	C18:3n3; Linolenic acid, methyl ester	21.736	415146	0.971
22	C20:0; Arachidic acid, methyl ester	24.687	642535	0.463
23	C20:1c; 11-Eicosenoic acid, methyl ester	25.273	70516	0.185
24	C20:2c; 11,14-Eicosadienoic acid, methyl ester	26.994	6027	0.013
26	C21:0; Heneicosanoic acid, methyl ester	28.397	20035	0.016
30	C22:0; Behenic acid, methyl ester	31.848	278788	0.203
31	C22:1n9; Erucic acid, methyl ester	32.705	56726	0.155
33	C23:0; Tricosanoic, methyl ester	35.067	35362	0.027
34	C24:0; Lignoceric acid, methyl ester	38.107	147829	0.107

Secondary metabolites

Standard identification procedures confirmed the presence of carbohydrates, saponins, tannins, flavonoids, terpenoids and phenols in the cladode as well as in the fruit part while terpenes were found in cladodes only. Similarly, alkaloids were detected in the fruit part only. Rest of the secondary metabolites were absent from both the parts.

The results have been displayed below (Table 3).

Table 3. Secondary metabolites of different parts of *Opuntia dillenii*.

Plant part	Crb[c]	Alk[d]	Sap[e]	Tan[f]	Fla[g]	Phlb[h]	Tpn[i]	Tpd[j]	Cgs[k]	Strls[l]	Phls[m]
O-Cl[a]	+++	---	+++	+++	+++	---	+++	+++	---	+++	+++
O-Fr[b]	+++	+++	+++	+++	+++	---	---	+++	---	---	+++

[a] = *Opuntia dillenii* cladodes; [b] = *Opuntia dillenii* fruit; [c]= Carbohydrates; [d] = Alkaloids; [e] = Saponins; [f] = Tannins; [g] = Flavonoids; [h] = Phlobatannins; [i] = Terpenes; [j] = Terpenoids; [k] = Cardiac glycosides; [l] = Sterols; [m] = Phenols; +++ = Present; --- = Absent.

Table 4. Effect of *Opuntia dillenii* extract at 100, 200 and 300 mg/kg i.p. in carrageenan-induced rat paw edema.

			Paw volume, ml					
Group	Treatment	Dose, Kg^{-1}	After administration					
			Normal	1 h	2 h	3 h	4 h	5 h
I	Saline (Control)	10 ml	1.0683±0.05	1.9650 ±0.09	2.0583 ±0.11	2.1667 ±0.08	2.2650 ±0.04	2.2983 ±0.03
II	Diclofenac	10 mg	1.1517±0.09	1.8000±0.10	1.6503 ±0.11	1.4633±0.19	1.5667±0.20	1.7550±0.14
III	Extract	100 mg	1.0867±0.13	1.9233±0.20	1.9617±0.21	1.9583±0.10	2.0450±0.06	2.0950±0.04
IV	Extract	200 mg	1.0200±0.08	1.7933±0.12	1.7633±0.11	1.6567±0.14	1.7567±0.12	1.7950±0.13
V	Extract	300 mg	1.1967±0.15	1.9333±0.11	1.8650±0.14	1.7367±0.14	1.8317±0.17	1.8950±0.15

Values are reported as mean ± S.E.M. n = 6. *P<0.05, **P<0.01 show statistically significant values from control.

Acute toxicity

The crude *Opuntia dillenii* extract remained safe at all dose levels of 250, 500 and 1000 mg/kg per orally. No toxic effects were observed, and the fed animals were found totally normal.

Anti-inflammatory activity

Anti-inflammatory activity of *Opuntia dillenii* crude extract was carried out at different dose levels (100, 200 and 300 mg/kg body weight) intraperitoneally. The results are given in Table 4 and Figure 1.

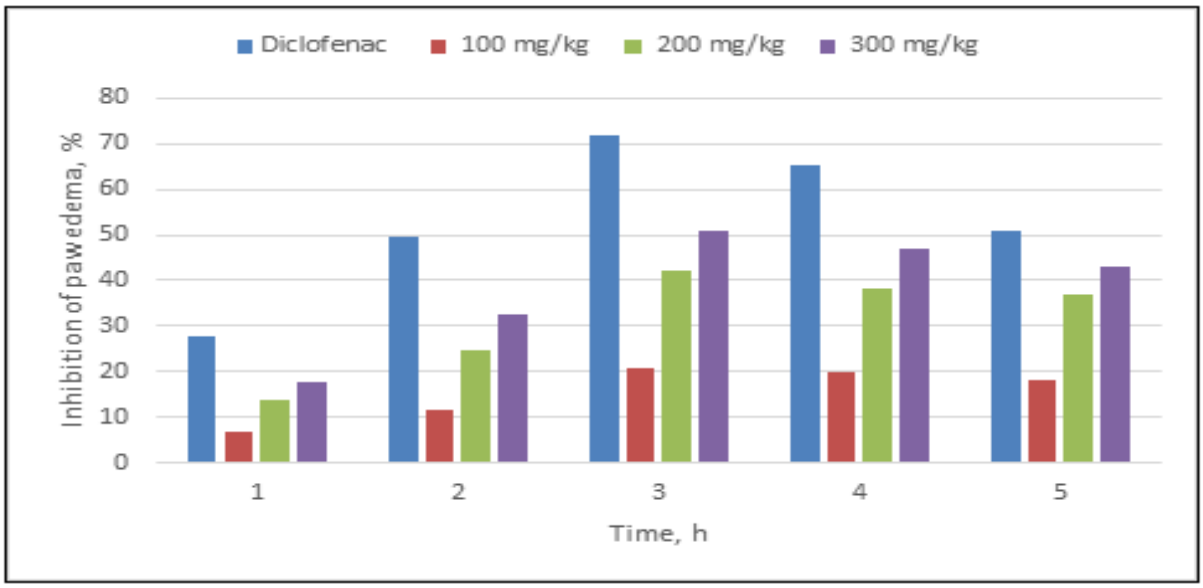

Fig. 1. Anti-inflammatory activity of *Opuntia dillenii* extract in carrageenan induced paw edema.

The data was analyzed by GraphPad Prism Ver. 6. Inflammation increased gradually after injecting carrageenan in the paw. The crude extract displayed significant that is, P< 0.01, anti-inflammatory activity (50.88 %) at a dose level of 300 mg/kg after 3 h dose injection. The standard used (diclofenac sodium, 10 mg/kg) displayed greater activity than our extract at different dose levels.

Conclusions

A thorough study confirmed the presence of carbohydrates, saponins, tannins, flavonoids, terpenoids, phenols, terpenes and alkaloids in *Opuntia dillenii* cladodes and fruit part. Linoleic acid, a potent cyclooxygenase-2 (COX-2) inhibitor, was the most abundantly found fatty acid (59.153 %) in the seeds of this plant. Metals analysis confirmed a high content for the minerals, potassium and calcium, in all the parts. Quite encouraging results were recorded for the anti-inflammatory activity of the crude extract of the cladodes part which may be due to different useful secondary metabolites present in the plant. No toxicity was observed for the extract while administering it to a 1000 mg/kg body weight dose level. The present study would hopefully help the researchers in the field to be directional from the right beginning towards their research interests on this astonishing plant.

Competing interests:

There are no competing interests.

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Ethics approval and consent to participate

The institutional guide was followed for the care and use of laboratory animals.

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