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Antioxidant potential and its relationship with polyphenol content and degree of polymerization in *Opuntia elatior* Mill. fruits

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Abstract

Background: *Opuntia elatior* Mill. (*Nagaphani*) fruits are traditionally recommended as an expectorant, remedy for whooping cough, asthma, gonorrhea, ulcers, tumors, in the treatment of diarrhea and syphilis. Many of these diseases are allied with oxidative stress caused by free radicals. Thus, current research is directed towards finding naturally-occurring antioxidants of plant origin. **Aim:** To evaluate antioxidant potential of hydro-alcoholic extract of the *O. elatior* fruits (HAOE) and its fractions. **Materials and Methods:** Using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide radical scavenging assay, total polyphenolic, flavonoid (FA), flavanone (FO) contents and degree of polymerization in relation with its antioxidant activity were examined. **Results:** The experimental data indicated that the HAOE, ethyl acetate (EAOE) and butanol (BFOE) soluble fractions have shown significant antioxidant activity. The highest polyphenolic, FA, FO contents and degree of polymerization were found in EAOE. The scavenging potential was in the order of Ascorbic Acid > EAOE > BFOE > HAOE > BIOE, where ascorbic acid was used as a positive control. The increased antioxidant potential of EAOE and BFOE fractions over HAOE extract may be attributed to the purification achieved by fractionation of the extract which in turn resulted in an increase in the degree of polymerization and segregation of secondary metabolites. **Conclusion:** The fruit of O. *elatior* can be used as the best alternative for synthetic antioxidants.

Key words: Antioxidant activity, flavanone, flavonoid, *Opuntia elatior*, polymerization

Introduction

About 5% or more of the inhaled oxygen is converted to reactive oxygen species (ROS) such as O_2, H_2O_2 and OH by univalent reduction of O_2.[1] Antioxidants can scavenge ROS by different mechanisms and plants are the potent source of natural antioxidants. The quest for natural antioxidants for dietary, cosmetic, and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades. *Opuntia elatior* Mill. (Family: Cactaceae) commonly known as Prickly pear and *Nagaphani*, is xerophytic subarborescent, three meter high ovate, oblong limbs. Fruits are berry pyriform, angular, bearing tufts of glochidia and occasionally a few prickles, reddish purple when ripe and greenish when unripe. It is indehiscent, many seeded pulpy fruit and depressed at the apex. Fruits are recommended as an expectorant and remedy for whooping cough, asthma, gonorrhea, ulcers, tumors, treatment of diarrhea and syphilis.[2]

Keeping the wide range of its therapeutic efficacies in view, the present study has been designed to evaluate the antioxidant potential and its relationship with polyphenol content and degree of polymerization in *O. elatior* fruits.

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Materials and Methods

Chemicals and standard drugs

Ascorbic acid, aluminum trichloride hexahydrate, Folin–Ciocalteu phenol reagent, naringin, gallic acid, rutin (hydrate, min 95%), 2,4-dinitrophenyl-hydrazine (DNPH)

and anisaldehyde (Analytical grade, S. D. Fine Chemicals Pvt. Ltd., Vadodara, Gujarat, India), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma–Aldrich Chemie, Steinheim, Germany), precoated silica gel G 60 F_{254} thin layer chromatography (TLC) aluminum plates (20 cm × 20 cm, 0.2 mm thick) (Merck Ltd., Germany) and AR grade chemicals were used.

Plant material, preparation of the extract and its fractionation

The plant was collected from the local region of Nagpur, botanically identified and authenticated at Department of Botany, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur, Maharashtra, India. A voucher specimen (specimen no. 9786) has been deposited for future reference.

The fresh fruits were pulverized and macerated with hydro-alcoholic (ethanolic) solvent (7:3). The hydro-alcoholic extract (HAOE) was concentrated in a rotary vacuum evaporator to yield a dark reddish-brown mass (yield: 10.5% w/w). For fractionation, HAOE was triturated with silica (1:3), loaded to Soxhlet assembly and extracted by ethyl acetate to yield ethyl acetate soluble fraction (EAOE; yield: 0.3% w/w). The ethyl acetate insoluble portion was further extracted with saturated n-butanol (7:3) to yield n-butanol soluble fraction (BFOE; yield: 26% w/w) and n-butanol insoluble fraction (BIOE; yield: 70% w/w). The HAOE and these three broad fractions, that is, EAOE, BFOE and BIOE were subjected to phytochemical and antioxidant screening.[3]

Phytochemical screening

The HAOE and its broad fractions were screened for the presence of unsaturated sterols, triterpenes, tannins, flavonoids (FA), saponins, carbohydrates and/or sugars with TLC. Thin layer plates precoated with silica gel G were used and development was carried out in the optimized solvent system "Toluene: Ethyl acetate: Methanol" (5:3:2, v/v/v). After the development of chromatogram in the solvents, plates were dried and sprayed with anisaldehyde-sulfuric acid, ferric chloride, and the p-anisidine hydrochloride reagent for the detection of saponins, tannins, carbohydrate and/or sugars, respectively. While, detection of FAs, protein/amino acids and unsaturated sterols was carried out using $AlCl_3$, hydroxylamine-ferric chloride, ninhydrin, and vanillin-sulfuric acid reagent, respectively, and visualization was carried out under visible and UV light (λ: 366 nm).[4]

Hydro-alcoholic extract and its fractions were also quantified for the presence of important secondary metabolites such as total polyphenol (TP), FA, and flavanone (FO) compounds using following spectroscopic methods.

Determination of total polyphenol

Total polyphenol content was measured using Folin–Ciocalteu colorimetric method.[5,6] Briefly, 0.1 ml of test sample was mixed with 1 ml of diluted Folin–Ciocalteu's phenol reagent (1:10 with distilled water; 0.2 N). After 3 min, 1 ml of saturated sodium carbonate (75 g/L) solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min and the absorbance was measured at 725 nm (Shimadzu UV–VIS spectrophotometer 1600) against the corresponding test and standard blanks prepared in the same way without the Folin–Ciocalteu's phenol reagent. Gallic acid was used as a reference for constructing a standard curve (20–100 mg/ml). The results were expressed as mg of gallic acid equivalents (GAE)/g of extract. All determinations were performed in triplicate.

Determination of flavonoids

Flavonoid content was determined by the aluminum chloride method.[7,8] Briefly, to 1 ml of test solution (1 mg/ml), 1.5 ml of 95% alcohol, 0.1 ml of 10% aluminum chloride hexahydrate ($AlCl_3.6H_2O$), 0.1 ml of 1 M sodium acetate (CH_3COONa) and 2.3 ml of distilled water were added. After incubation at room temperature for 40 min, absorbance of the reaction mixture was measured at 435 nm against corresponding blank, prepared in the same manner without adding $AlCl_3$. Rutin was used as a reference standard (20–100 mg/ml), and results were expressed as mg of rutin equivalents (REs)/g of extract. All determinations were performed in triplicate.

Determination of total flavanones

The modified DNPH method was used for determination of FOs.[9] Naringin was used as the reference standard. Twenty milligrams of naringin was dissolved in methanol and then diluted to 500–2500 μg/ml. One milliliter of each of the diluted standard solutions was reacted separately with 2 ml of 1% DNPH reagent and 2 ml of methanol at 50°C for 50 min. After cooling at room temperature, the reaction mixture was mixed with 5 ml of 1% KOH in 70% methanol and incubated at room temperature for 2 min. Then, 1 ml of the mixture was taken, mixed with 5 ml of methanol and centrifuged at 1000 × *g* for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 ml. The absorbance of the supernatant was measured at 495 nm. All test samples were similarly reacted with DNPH for determination of FOs. The mean of three readings was used, and the results were expressed as mg of naringin equivalents (NE)/g of extract.

In vitro antioxidant assays

1,1-diphenyl-2-picrylhydrazyl radical scavenging assay

The free radical scavenging activity was evaluated by the DPPH assay.[10] In its radical form, DPPH has absorbance maxima at 517 nm, but upon reduction by an antioxidant, the absorbance decreases. Briefly, 1 ml of 0.25 mM solution of DPPH in methanol was added to 1 ml of HAOE/EAOE/BFOE/BIOE solution in methanol (20–100 μg/ml). After 20 min, the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The percentage DPPH decolorization of the sample was calculated by the following equation. All determinations were made in triplicates.

$$\% \text{ of DPPH scavenging} = ([A_{control} - A_{extract}]/A_{control}) \times 100$$

Where A is the absorbance.

Nitric oxide radical scavenging assay

The nitric oxide (NO) radical inhibition activity was measured using Griess reagent with little modifications.[11] Briefly, the reaction mixture containing, 4 ml of sodium nitroprusside (5 mM), 1 ml phosphate buffer saline (pH 7.4) and 1 ml of test sample or standard solution at various concentrations (20–100 μg/ml) was incubated at 25°C for 150 min. After incubation, 1 ml of

the reaction mixture containing nitrite was removed, mixed with 4 ml of Griess reagent (0.1% w/v, N-(1-naphthyl) ethylenediamine dihydrochloride and sulfanilic acid [0.33% w/v; 1:1]) and allowed to stand for 30 min. A red-violet colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solution in triplicates. The percentage NO inhibition of the sample was calculated by the equation.

$$\% \text{ of NO inhibition} = ([A_{control} - A_{extract}]/A_{control}) \times 100$$

Where A is the absorbance.

Observations and Results

Phytochemical screening

Qualitative phytochemical screening revealed the presence of carbohydrates, steroid, FA, proteins and tannins in HAOE extract. EAOE, BFOE fractions have shown the presence of carbohydrates, sterols, FAs and tannins. However, BIOE showed the prominent presence of carbohydrates and very minute color intensity was found in the case of FAs and tannins screening [Figure 1].

Determination of total polyphenol, flavonoids, flavanone compounds, and degree of polymerization

The TP content (mg/g) was found in a range of 11.06–72.83 GAE mg/g of extract and the highest content of polyphenolic compounds was found in EAOE fraction (72.83 ± 0.111 GAE mg/g of extract) [Table 1]. Polyphenol content was determined from linear regression equation of gallic acid and expressed as GAE of extract ($y = 0.010x + 0.005$, $r^2 = 0.992$) [Figure 2].

The FA content varied from 5.72 to 15.06 RE mg/g of extracts [Table 1]. FA content was ascertained from linear regression equation of Rutin ($y = 0.005x + 0.043$, $r^2 = 0.974$) [Figure 2].

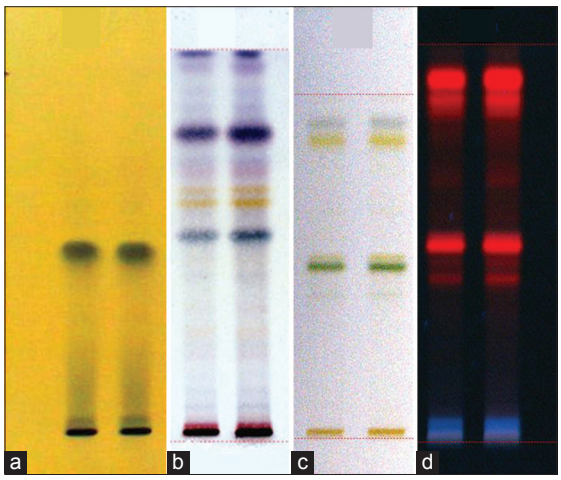

Figure 1: Graphical representation of phytochemical screening for the extracts/fractions. (a) Sprayed with ferric chloride solution at visible light; (b) sprayed with vanillin-sulfuric acid solution at visible light; (c) sprayed with p-anisidine-hydrochloride solution at visible light; (d) sprayed with aluminum trichloride solution at 366 nm

The FO content was examined by DNPH method found in between 0.3 and 2.2 NE mg/g of extract [Table 1]. FO content was determined from linear regression equation of naringin ($y = 0.0024x + 0.0188$, $r^2 = 0.981$) [Figure 2].

The flavones, flavonols, and isoflavones formed complexes only with aluminum chloride, while FOs strongly reacted only with DNPH, so the contents determined by the two methods were added up to obtain the total FA content (TFA).[12]

Degree of polymerization

The FA and FO contents represented 23.18% (w/w) and 2.38% (w/w) of the TP in HAOE respectively and similar pattern was observed in all its fractions, suggesting that the extracts are very complex, contain many other polyphenols such as coumarins, phenolic acids, and tannins. The degree of polymerization of the polyphenols present in the samples is substantial and can be estimated by the ratio between the TP and TFA contents. The highest degree of polymerization was observed in EAOE fraction (4.21 ± 0.061), and it varies from 1.83 to 4.21 [Table 1].

In vitro antioxidant assay

Antioxidant activity was carried out using DPPH and NO assay. Analysis of the free radical scavenging activities of the extracts revealed a concentration-dependent antiradical activity resulting from the reduction of DPPH, NO radicals to nonradical form. The extract HAOE and fractions EAOE and BFOE have shown significant antioxidant activity.

1,1-diphenyl-2-picrylhydrazyl method

The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals was initiated by the lipid autoxidation. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Positive DPPH test suggests that the samples were free radical scavengers.

While comparing with the standard, amongst the extract and its fractions, EAOE has shown most prominent antioxidant activity with the IC_{50} value 44.52 ± 0.531 µg/ml. The

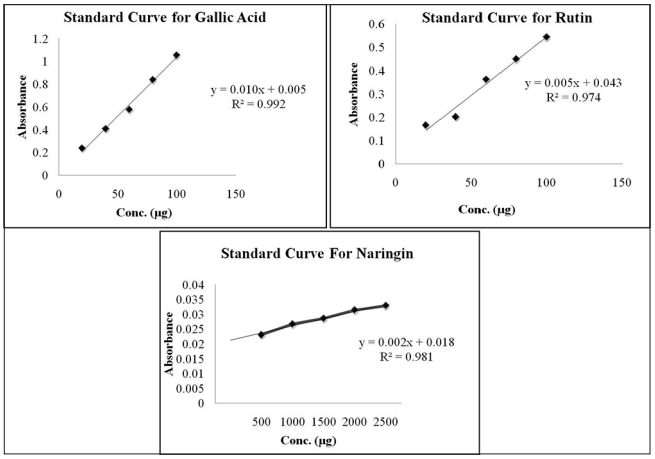

Figure 2: Graphical representation of standard linearity curves for gallic acid, rutin, and naringin

Table 1: Antioxidant potential and contents of TP, FA, FO in HAOE extract and its fractions of *Opuntia elatior* fruit

Extract/ fractions	DPPH assay IC_{50} value	NO assay IC_{50} value	TP (GAE mg/g of extract)	FA (RE mg/g of extract)	FO (NE mg/g of extract)	TFA[a]	Polymerization degree[b]
HAOE extract	64.14±0.18	81.43±0.70	44.00±0.10	10.20±0.01	1.05±0.00	11.25±0.03	3.91±0.09
EAOE fraction	44.52±0.53	51.08±0.19	72.83±0.11	15.06±0.09	2.20±0.00	17.26±0.12	4.21±0.06
BFOE fraction	57.28±0.21	75.27±0.28	60.20±0.60	12.60±0.14	1.90±0.00	14.5±0.4	4.15±0.00
BIOE fraction	136.36±0.31	143.40±0.18	11.06±0.08	5.72±0.02	0.30±0.05	6.02±0.10	1.83±0.11

Results are means±SD of three replicates: GAE, RE and NE, respectively; [a]TFA is determined by adding FA content with FO content; [b]The estimation of the polymerization degree was calculated by the ratio between TP and TFA. SD: Standard deviation, TP: Total phenolics, FA: Flavonoid, FO: Flavanones, HAOE: Hydro-alcoholic extract, DPPH: 1,1 diphenyl-2-picrylhydrazyl, NO: Nitric oxide, GAE: Gallic acid equivalents, RE: Rutin equivalents, NE: Naringin equivalents, TFA: Total flavonoid

scavenging activity of ascorbic acid, a known antioxidant, used as positive control, was higher and scavenging potential was found to be in the following order of ascorbic acid > EAOE > BFOE > HAOE > BIOE [Figure 3a].

Nitric oxide method

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by the extract/fractions. The EAOE at varying concentrations showed remarkable inhibitory effect of NO radical, comparative to HAOE, BFOE and BIOE. Results showed the percentage of inhibition in a dose-dependent manner for all the extract/fractions tested. The concentration of EAOE needed for 50% inhibition (IC_{50}) was found to be 51.08 ± 0.197 µg/ml. The NO scavenging activity of ascorbic acid was found similar as that of DPPH method and scavenging potential was found in the order of Ascorbic acid > EAOE > BFOE > HAOE > BIOE [Figure 3b].

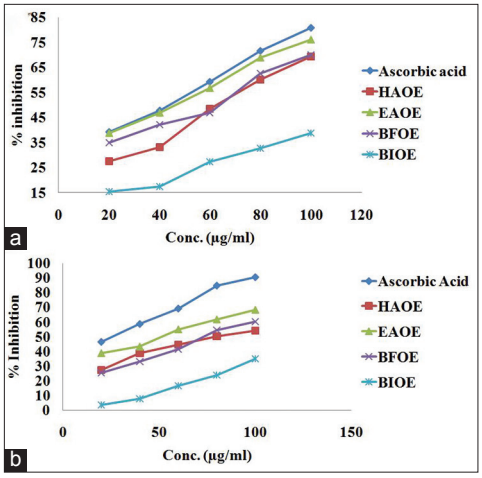

Figure 3: Graphical representation of antioxidant activity for the extracts/fractions. (a) 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay; (b) nitric oxide radical scavenging assay

Discussion

In living systems, free radicals are constantly generated; few amongst those remain as the unregulated radicals, which can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis.[13] The phenolic compounds are very important plant constituents because of their scavenging ability by virtue of hydroxyl groups and are, therefore, known as powerful chain-breaking antioxidants. These compounds may contribute directly to antioxidant action and it is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1 g daily from a diet rich in fruits and vegetables.[14] The results indicate that EAOE had the highest antioxidant activity, which may be due to the presence of the highest concentration of polyphenols and FAs. The *Opuntia elatior* fruits are already reported to contain betacyanin content (47.10 mg/100 ml) equivalent to betanin pigment.[15] The betanin pigment has been demonstrated in a wide range of assays for its antioxidant properties,[16] and it was reported that it effectively increased resistance to oxidation.[17] The increased antioxidant potential of EAOE and BFOE fractions over HAOE extract may be attributed to the purification achieved by fractionation of extract, which in turn resulted in increase in degree of polymerization and segregation of secondary metabolites such as steroids, complex polyphenols [Figure 3a and b] and may be betanin pigments. However, the lowest degree of polymerization and antioxidant activity of BIOF may be because of the negligible amount or the absence of potential secondary metabolites [Figure 3a and b] [Table 1].

Conclusion

Opuntia elatior showed strong antioxidant activity by inhibiting DPPH and NO activities when compared with standard L-ascorbic acid. In addition, all the extracts were found to contain a noticeable amount of total phenols, FA and FO which play a major role in controlling oxidation. The potent scavenging potential and a high degree of polymerization and segregation of secondary metabolites in EAOE and BFOE fractions comparative to HAOE were observed. These results are in agreement with previous studies; where, with an increase in the degree of polymerization of extract, cytotoxic, anti-inflammatory, and immunomodulatory activity also increases.

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हिन्दी सारांश

नागफणी फल के हाईड्रोअल्कोहोलिक सत्व की एंटीऑक्सीडेंट क्षमता का मूल्यांकन

प्रकाश आर. इटनकर, वर्षा ए. सोनटक्के, मोहम्मद ताकिर, सोनल एस. चरडे

नागफणी (ओपुनशिया इलाटिओर) फल पारंपरिक दौर से काली खांसी, श्वास मे कफनिसारक रूप से तथा सूजाक, अल्सर, ट्यूमर में उपयोगी है, तथा इस का उपयोग दस्त और उपदंश के इलाज मे भी होता है। इन में से कई रोगों की वजह ओक्सिडेटिव तनाव से निर्मीत मुक्त कण है। वर्तमान शोध संयंत्र वनस्पतीयों मे स्वाभाविक रूप से होने वाली एंटीऑक्सीडेंट गतिविधि खोजने की दिशा में निर्देशित किया गया है। प्रस्तुत अध्ययन नागफणी फल के हाईड्रोअल्कोहोलिक सत्व की एंटीऑक्सीडेंट क्षमता का मूल्यांकन करने के लिए किया गया। परिणामों में नागफणी फल के हाईड्रोअल्कोहोलिक सत्व की एंटीऑक्सीडेंट गतिविधि के सार्थक परिणाम प्राप्त हुये। नागफणी का फल, सिंथेटिक एंटीऑक्सीडेंट के लिए सबसे अच्छा विकल्प के रूप में इस्तेमाल किया जा सकता है।