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Evaluation of Phytochemical constituents and Antioxidant Activity of *Opuntia ficus-indica* L. cladode extracts

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ABSTRACT: *Opuntia ficus-indica* L. (Cactus), commonly referred to as prickly pear or nopal cactus, is an excellent source of minerals, dietary fiber, vitamins and various phytochemicals. The present study is aimed to assess the phytochemical analysis and antioxidant potential of cladode extracts of *Opuntia ficus indica* L. Phytochemical analysis showed the presence of significant amount of phenolics, flavonoids and tannins in cladode extracts. Among all the tested extracts, acetone cladode extract showed highest phenolic content of 25.20 ± 0.30 mg GAE/gm of dry sample. Acetone cladode extract showed highest flavonoid content of 10.30 ± 1.11 mg CE/g of dry samples. Methanolic cladode extract was found to contain highest tannin content of 7.80 ± 0.24 mg GAE/g of dry samples. The antioxidant activity of the extracts was determined by DPPH free radical scavenging method. The highest antioxidant activity was recorded in ethanol and acetone cladode extract with the percentage inhibition of 78.27 ± 1.22 and 57.90 ± 1.78 respectively. Six different compounds with antioxidant potential were isolated from the acetone, methanol, ethanol and petroleum ether extracts using thin layer chromatography. GC-MS analysis of acetonic cladode extract showed the presence of Hexadecanoic acid (synonym – Palmitic acid). Compound Limonene dioxide 2 was found in methanol cladode extract. Ethanol extracts showed the presence of 1) 3, 5-Bis (trimethylsiloxy) benzoic acid, trimethylsilyl ester 2) 1, 2- Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate and 3) Hydrocinnamic acid, benzyldimethylsilyl ester as an antioxidant compound. 5,7 dimethyloctahydrocoumarin 1 was found as an antioxidant in petroleum ether extract. The challenge in the present study was to isolate these antioxidant compounds in pure form, but thin layer chromatography based antioxidant assay by using DPPH method resulted in to better isolation of these bioactive compounds. The present study revealed and identified the bioactive compounds from opuntia *Ficus indica* L. cladodes which have potential as a strong antioxidant and can be key to the discovery of new antimicrobials.

Keywords: Cactus, Opuntia, Cladode, Antioxidant.

INTRODUCTION

Opuntia ficus-indica (Cactus) commonly referred to as prickly pear or nopal cactus, is a member of the Cactaceae family. Cactus is a tropical or subtropical plant originally grown in South America for utilization as a fruit and forage crop (Butera *et al.,* 2002). Cladodes of *opuntia* plant are highly nutritious and are widely consumed as staple food (Corrales-Garcia *et al.,* 2004). *Opuntia ficus indica* has been proved useful in the treatment of diabetes mellitus, hyperlipidemy, obesity and gastrointestinal disorders (Corrales-Garcia *et al.,* 2004). *Opuntia* cladodes possess significant cicatrizing, anti-inflammatory, pain relief, antiviral and antioxidant activities and hence they has been used extensively in folk medicine (Park *et al.,* 2001). Moreover, cactus cladodes also found to protect against many chronic diseases, including cancer, cardio, cerebro-vascular and neurological diseases (Zou *et al.,* 2005). Since the cladodes of *opuntia* possesses a great number of bioactive phytochemicals like calcium, fibers and phenolics they have been consumed as vegetables to promote the health (Ayadi *et al.,* 2009). It is also a rich source of polyphenols and flavonoids which are responsible for health benefits. Ascorbic acid, a natural antioxidant, is also found in considerable quantities in *Opuntia* cladodes (Stintzing and Carle 2005). Antioxidants are the chemical components with the capacity of scavenging free radicals (Tsuda *et al.,* 1996). Various compounds like polyphenols, Carotenoids, tocopherol, ascorbic acid and flavonoids have been reported for their antioxidant properties (Wang *et al.,* 2011). Synthetic antioxidant compounds have some serious side effects since they possess allergenic and carcinogenic properties. They can cause nausea and may be responsible for DNA and sperm

abnormalities etc. Due to these safety issues, the demand for natural antioxidants has increased globally (Wojcik *et al.,* 2010). Moreover, synthetic antioxidants do not contribute additional nutritional benefits (Satyanarayana *et al.,* 2014). One of the best alternatives to these synthetic antioxidants is the use of natural antioxidants. The main benefit of using these natural antioxidants is that they are easily assimilated by body with negligible or no side effect (Wojcik *et al.,* 2010). In addition, there is also the challenge of multi-drug resistance to available antimicrobials (Valtierra-Rodríguez *et al.,* 2010). All these challenges have forced the scientific world to investigate plants for presence of novel and safe bioactive compounds possessing natural antioxidant and antimicrobial properties (Valtierra-Rodríguez *et al.,* 2010). *Opuntia ficus indica* L. is one of the plant that is being investigated by the researchers for the presence of natural antioxidants and antimicrobial compounds. Several studies on *Opuntia* spp. have reported the antioxidant (Albano *et al.,* 2015) and antimicrobial (Gnanakalai and Gopal 2016) activities and it is attributed to the presence of polyphenol compounds in the *Opuntia* spp. (Livrea and Tesoriere 2016).

MATERIAL AND METHODS

Collection of plant material. The *Opuntia ficus-indica* L. cladodes were collected from the Bor forest region of Wardha district (MS), India. The sample collecting site, Bor forest region is located near Hingani in Wardha district; while the district of Wardha is located between *latitude* 20°-21°N *and longitude* 78°-79°E of Maharashtra state. The plant was authenticated at Department of Botany Bajaj College of Science, Wardha (MS) India.

Sample preparation. Freshly collected cladodes from *Opuntia ficus-indica* L. were washed with water and disinfected by using 10% sodium hypochlorite solution. Cladodes were sliced into small pieces in order to facilitate the drying process. Cladodes were allowed for the complete shade drying in an open room at room temperature for three weeks. Finally, the dried cladodes were pulverized to fine powder and stored at room temperature until further analysis.

Preparation of plant extracts. The dried powder was filled in a bag (25g) made up of Whatman No. 1 filter paper and was subjected to Soxhlet extraction with 200 ml of solvents with rising polarity index such as petroleum ether, acetone, ethanol and methanol in the ratio of 1:6 w/v in at 45-50°C until the extract was clear or colourless. Extraction was carried out in controlled conditions of temperatures to prevent the loss of heat sensitive phytochemicals. The resulting extracts were concentrated in a rotary evaporator under reduced pressure at 40°C. Dried extracts were weighed in an analytical balance. The extracted materials were stored at 4°C until use.

Estimation of Total Phenolic Content. Total phenolic content of different solvent extracts of cladodes were determined spectrophotometerically according to the Folin-Ciocalteau colorimetric method (Singleton and Rossi 1965). A volume of 200 µl of each extract was taken into screw cap test tubes and 1.0 ml of Folin-Ciocalteau reagent (1:1 with water) and 1.0 ml of sodium carbonate (7.5%) were added in to it. The contents were mixed by vortexing the tubes and tubes were incubated for 2 hrs. After that, absorbance was read at 726 nm using a spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material.

Estimation of Total Flavonoid Content. Aluminium chloride colorimetric method was used for determination of total flavonoid content of cladode extracts (Lin and Tang 2007). Each cladode extract (2 ml, 0.3 mg/ml) in methanol was mixed with 0.1 ml of 10% aluminium chloride hexahydrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and incubated for 40 minutes at room temperature. After incubation, absorbance was read at 415 nm. Quercetin was used as a standard (the concentration range: 0.005 to 0.1 mg/ml) and the total flavonoid content was expressed as milligram CE per gram of dry extracts.

Estimation of Tannin content. Quantitative estimation of tannins in each cladode extract was carried out by using modified vanillin–HCl in methanol method (Price and Bulter 1977). This method is based on the ability of condensed tannins to react with vanillin in the presence of mineral acid to form a red color. Each cladode extract (1 ml) was mixed with 20 ml of 1% HCl (in methanol) for 20 min at 30°C in a water-bath. The samples were centrifuged at 2000 rpm for 4 min. After centrifugation, supernatant (1 ml) was mixed with 5 ml vanillin solution (0.5% vanillin + 2% HCl in methanol) for 20 min at 30°C. Blanks were run with 4% HCl in methanol in place of vanillin reagent. Absorbance was taken at 500 nm on a UV spectrophotometer. A standard curve of with catechin was prepared. Results were expressed as mg of gallic acid equivalent (mg GAE)/gm of dry extracts. Samples were analyzed in triplicate.

Antioxidant Activity (DPPH radical scavenging assay). Antioxidant activity of *opuntia* cladodes was determined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay method. In carrying out the DPPH assay, 10 µl of each extract and 190 µl of methanolic solution of 0.1 mM DPPH were mixed. The mixture was then shaken vigorously and incubated at 37° C for 5 min. The absorbance was measured at 517 nm on ELISA plate reader. The blank was 80% (v/v) methanol, and DPPH in methanol was used as the negative control. Ascorbic acid was used as a positive control. The percentage DPPH inhibition was calculated using the following formula:

$$I\,(\%) = (A0 - A1)/A0 * 100$$

Where A0 = absorbance of negative control, A1 = absorbance of the extract. The experiment was performed in triplicate. The percentage radical scavenging activity versus extract concentration curve was plotted and the concentration of the sample that was required for 50% radical scavenging activity was determined and expressed as the IC50 value. Lower IC50 values indicated high antioxidant capacity (Grzegorczyk *et al.,* 2007).

Fractionation of compounds from cladode extracts. Thin layer chromatography was performed using silica

gel coated TLC plates to separate the number of compounds present in the given extract. A 5 µl (1 mg/ml) sample of each extract was spotted at 1 cm from the bottom of plates by using capillary tubes. For separation of metabolites from extract, solvent system was optimized by using different solvents at various combinations and concentrations. TLC plates were kept under pre-saturated (eluent vapour) chromatographic chamber for the development of chromatogram. The developed chromatogram was visualized under UV light (365 nm and 254 nm) and using iodine vapour. The Rf values of the compounds were calculated using the following formula.

$$Rf = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent front}}$$

TLC based Antioxidant Activity. An aliquot of active extracts of *Opuntia ficus-indica* L. (1 mg/ml) was spotted onto the TLC plates. The TLC plates were developed in solvent saturated chamber with solvent systems. After development, plates were air dried for 30 minutes. For detection of antioxidant compounds, DPPH solution (10 mg/ml) was sprayed over the TLC plate and kept few minutes for drying. While drying, a pale yellow spot observed on the TLC plate in the vicinity of the dispersed compounds. The Rf values for the antioxidant compounds were calculated.

Identification of bioactive compounds by GC-MS Analysis. The TLC bands with the antioxidant activity were scraped and dissolved in a 1 ml methanol solvent (HPLC grade), centrifuged at 3000 rpm for 15 minutes. The supernatant was transferred to the sample tube, without disturbing the settled silica particles and used as a test sample. Sample volume of 2 µl was used for GC-MS analysis.

RESULTS

Quantitative estimation of total phenolics, flavonoid and tannin content. The results of total phenolic content, flavonoid content and tannin content of *Opuntia ficus indica* L. cladodes are presented in Table 1. Results of total phenolic contents are expressed as milligram GAE (Gallic Acid Equivalent) per gram of dry extract and total flavonoid content are expressed as milligram CE (Catechin Equivalent) per gram of dry extract. Acetone, ethanol and methanol solvents exhibited maximum capacity to extract the phenolic compounds than petroleum ether solvent. Among all the tested extracts, acetone cladode extract showed highest phenol content of 25.20 ± 0.30 mg gallic acid equivalent/gm of dry sample. Methanolic cladode extract showed phenol content of 21.40 ± 1.30 mg gallic acid equivalent/gm of dry sample. Phenol content of ethanolic cladode extract was found to be 20.10 ± 1.00 gallic acid equivalent/gm of dry sample. In contrast, petroleum ether extract showed lowest phenol content of 8.10 ± 0.10 mg gallic acid equivalent/gm of dry sample. The present study states that, petroleum ether was the poor source of phenolic compounds as compared to other extracts. Flavonoids content of the given samples was spectrophotometrically calculated. Of the four different solvent extracts of *Opuntia ficus indica* L. cladodes studied, the amount of flavonoids was found in the order of Acetone (10.30 ± 1.11) > Methanol (7.20 ± 0.40) > Ethanol (6.30 ± 0.33) > Petroleum ether (4.32 ± 1.20) mg CE/g of dry samples respectively. It was evident from the study that, among the various cladode extracts of *Opuntia ficus indica* L., tested acetone exhibited higher TFC value. Methanolic cladode extracts showed the highest tannin content of 7.80 ± 0.24 mg GAE/g of dry samples. Acetone and ethanol cladode extracts showed the tannin content of 6.20 ± 0.30 and 5.20 ± 0.32 mg GAE/g of dry samples respectively. On the other hand, petroleum ether extract was found to exhibit lowest 4.40 ± 0.10 mg GAE/g of dry samples.

Antioxidant activity. In this study, the cladode extracts of *Opuntia ficus indica* L. were examined for their scavenging of DPPH. The results of DPPH free radical scavenging activity of *Opuntia ficus indica* L. cladode extracts are presented in Table 2. The ethanol cladode extract showed highest antioxidant activity of 78.27 ± 1.22% (IC50= 5.24) followed by acetone cladode extract 57.90 ± 1.78% (IC50= 8.88), Petroleum ether cladode extract 40.62 ± 1.26 (IC50= 12.74), methanol cladode extract 20.45 ± 1.69 (IC50= 25.76). Overall, ethanol cladode extract exhibited strong antioxidant potency as compared to others and found to be lesser when compared with standard ascorbic acid.

Isolation and characterization of antioxidant compounds

TLC analysis. All the four extracts were studied for isolation and identification of bioactive compounds. TLC plate was developed using different solvent systems such as chloroform: ethyl acetate: methanol, petroleum ether: ethyl acetate: methanol, hexane: ethyl acetate: methanol, chloroform: methanol: petroleum ether. Among all the solvent systems used chloroform: ethyl acetate: methanol (9:6:2) was used as eluent for achieving greatest degree of separation of active compounds from ethanolic and methanolic cladode extracts. Highest degree of separation of active compounds from acetone and petroleum ether cladode extracts were achieved by using petroleum ether: ethyl acetate: methanol (10:5:2) as eluent. The Rf values of separated components on TLC plates were calculated by dividing the distance travelled by the compound of interest with the distance travelled by the solvent front. The calculated Rf values for acetonic cladode extract showed the peaks at Rf values in range of 0.25-0.74. Methanol cladode extracts showed the peak at Rf value in the range of 0.16-0.80. Petroleum ether cladode extracts showed the peak at Rf value in the range of 0.05-0.45. Ethanol cladode extracts showed the peak at Rf value in the range of 0.24-0.93. Maximum four components were observed in acetone cladode extract, six components in methanol cladode extract, seven in ethanol cladode extract and 3 in petroleum ether cladode extract.

TLC based antioxidant assay. The developed TLC plates sprayed with DPPH were examined for yellow bands on a purple background representing compounds with antioxidant activity. In our study, all the tested extracts showed antioxidant ability but number of active compounds was varied. The components with

antioxidant activity were detected at different Rf values in acetone cladode (0.74), methanol cladode (0.61), petroleum ether cladode (0.05), ethanol cladode (0.24, 0.31, 0.93) extracts.

GC-MS analysis. The fraction showing antioxidant activity was subjected to GC-MS analysis for the identification of compounds. The results of GC-MS analysis revealed that the six different compounds with antioxidant potential were found to be present in *Opuntia ficus indica* L. cladode extracts (Fig. 1-6). The compound Hexadecanoic acid was detected in acetonic cladode extract, while compound limonene di oxide 2 was recorded in methanolic cladode extract. The compound with antioxidant activity from the petroleum ether cladode extract was characterized as 5, 7 dimethyloctahydrocoumarin 1. However, three different compounds responsible for exhibiting strong antioxidant effect in ethanolic extract of cladode were identified as 1) 3,5-Bis (trimethylsiloxy) benzoic acid, trimethylsilyl ester 2) 1,2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate and 3) Hydrocinnamic acid, benzyldimethylsilyl ester. These compounds may have a major contribution in exhibiting significant antioxidant activity.

DISCUSSION

In the present study, acetone ethanol and methanol solvent extracts showed maximum capacity to extract the phenolic compounds from cladodes than petroleum ether. As per the previous studies it has been proved that the antioxidant capacity of plant extracts is strongly associated with their polyphenol content (Skerget *et al.,* 2005). Among the various phytochemicals, phenolics have been found to be the crucial plant compounds with antioxidant potential attributable to their redox properties, which facilitates the neutralization of free radicals (Zheng and Wang 2001). Several studies revealed that antioxidant capacity of plant extracts was moderately high which are rich in flavonoids (Cakir *et al.,* 2003).

The phytochemical contents detected in cladode extracts are known for their medicinal values and phenolic compounds derived from plants show biological activities like antioxidant (Espinosa *et al.,* 2015) and antifungal (Zabka and Pavela 2013). Few papers have been reported on the antioxidant activity and phenolic composition of cladodes of *O. ficus-indica* grown in the overseas. As per the report of Dib *et al.* (2013) the composition of phenolic compounds of cladodes of *O. ficus-indica* is found to be: total polyphenols 26.7±0.2 (mg GAE/g DW), flavonoids 11.9 mg CE/g DW and total tannin 6.45±0.11 (mg GAE/g DW).

However, it is important to underline that differences in TPC could arise from different climatic conditions. In particular, looking at *Opuntia* spp. plants, all parts of the cactus are particularly rich in polyphenolic classes, such as various flavonoids and phenolic acids, as reviewed by El-Mostafa *et al.* (2014).

In the present study, the compound Hexadecanoic acid (synonym - Palmitic acid) was detected in acetonic extract of cladodes. Wright and Setzer (2013) studied the chemical composition of volatiles from *Opuntia ficus-indica* and reported the dominance of palmitic acid (12.7 %) in the essential oil of *O. ficus-indica cladodes*. Total three different compounds with antioxidant activity were characterized from the ethanolic cladode extract. First compound is characterized as 1, 2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate. Salem *et al.* (2016) reported the presence of similar compound 1, 2-benzenedicarboxylic acid, dioctyl ester from dichloromethane extracts of wood branch resulted from the tree pruning wastes of *Schinus molle* L. grown in Egypt. Second compound from ethanolic cladode extract is identified as 3, 5-Bis (trimethylsiloxy) benzoic acid, trimethylsilyl ester. This compound is the derivative of benzoic acid. The related compound viz. 3,4-Dihydroxybenzoic acid and 4-hydroxybenzoic acid was identified in cladodes of *O. ficus-indica* (El-Mostafa *et al.,* 2014). Third compound is characterized as Hydrocinnamic acid, benzyldimethylsilyl ester as an antioxidant compound. This compound belongs to the class of phenolic compounds. Phenolic acids are split into two subgroups: hydroxybenzoic and hydroxycinnamic acids (Clifford 1999). Hydroxycinnamic acids are derivatives of cinnamic acid. The most abundant hydroxycinnamic acids in *O. ficus-indica* are cinnamic, chlorogenic, coumaric, and ferulic acids. Lower concentrations of caffeic, sinapic, and dimethoxycinnamic acids were reported (Ouerghemmi *et al.,* 2017). From methanol cladode extract Limonene dioxide 2 was identified as antioxidant compound. A similar compound viz. limonene was reported by Zito *et al.* (2013) from essential oils of two Sicilian cultivars of *Opuntia ficus indica L.*

A compound 5, 7 dimethyloctahydrocoumarin 1 was found as an antioxidant in petroleum ether extract. A related compound, Octahydrocoumarin 5, 7-dimethyl have been reported from the flowers of *M. aurea* by Kheder *et al.* (2014).

Table 1: Total phenolic, flavonoid and tannin content of *Opuntia ficus indica* L. cladode extracts.

Extract	Total Phenol Content (mg GAE/g of dry samples)*	Total Flavonoid Content (mg CE/g of dry samples)*	Tannin content (mg GAE)/g of dry samples
Acetone	25.20 ± 0.30	10.30 ± 1.11	6.20 ± 0.30
Methanol	21.40 ± 1.30	7.20 ± 0.40	7.80 ± 0.24
Ethanol	20.10 ± 1.00	6.30 ± 0.33	5.20 ± 0.32
Petroleum Ether	8.10 ± 0.10	4.32 ± 1.20	4.40 ± 0.10

Note: GAE- Gallic Acid Equivalent; CE- catechin. *Values are means of triplicate with standard deviations.

Table 2: Antioxidant activity of *Opuntia ficus indica* L. cladode extracts by DPPH assay.

Extract	Antioxidant activity (%)	IC 50
Ethanol	78.27 ± 1.22	5.24
Acetone	57.90 ± 1.78	8.888
Petroleum ether	40.62 ± 1.26	12.74
Methanol	20.45 ± 1.69	25.76
Ascorbic acid	84.33±2.20	2.74

Note: @10 mg sample/assay; *values are means of triplicate with standard deviations.

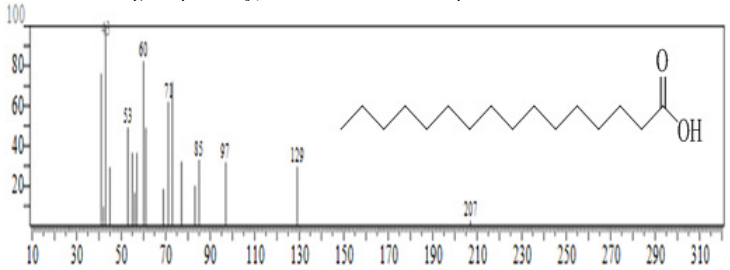

Fig. 1: Compound Hexadecanoic acid identified in acetonic cladode extract of *Opuntia ficus indica* L.

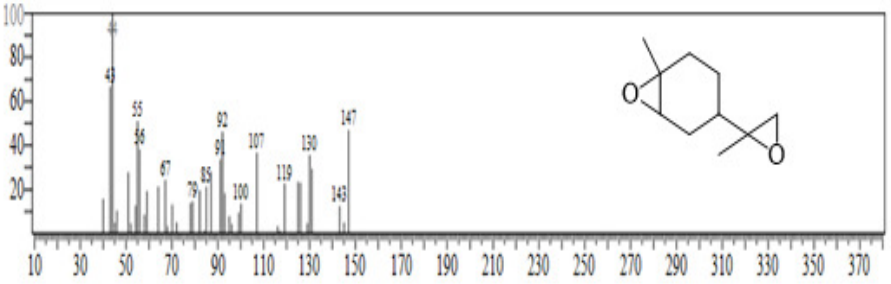

Fig. 2: Compound Limonene dioxide 2 identified in methanolic cladode extract of *Opuntia ficus indica* L.

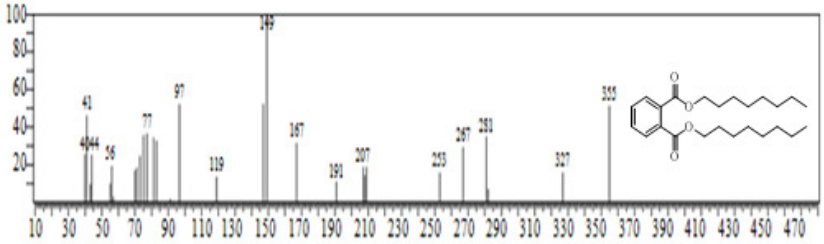

Fig. 3. Compound 1,2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate identified in ethanolic cladode extract of *Opuntia ficus indica* L.

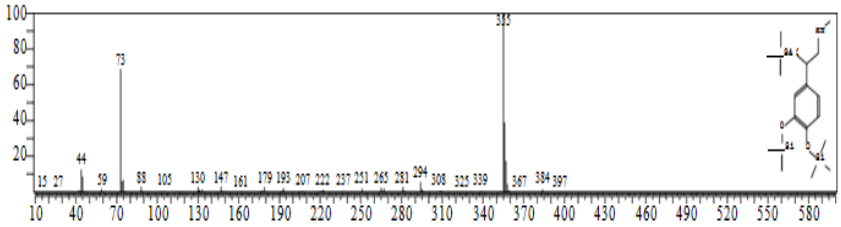

Fig. 4. Compound 3, 5-Bis (trimethylsiloxy) benzoic acid, trimethylsilyl ester identified in ethanolic cladode extract of *Opuntia ficus indica* L.

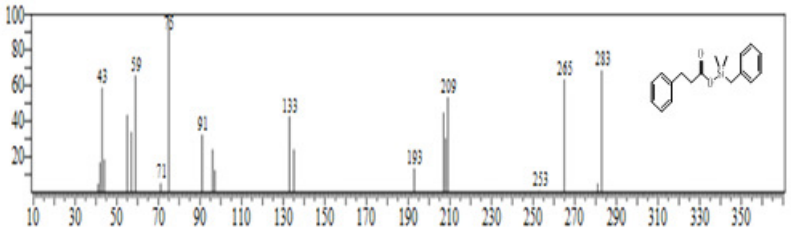

Fig. 5. Compound Hydrocinnamic acid, benzyldimethylsilyl ester identified in ethanolic cladode extract of *Opuntia ficus indica* L.

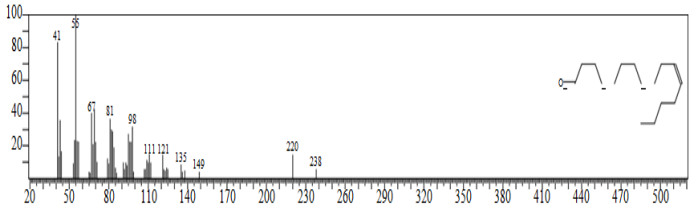

Fig. 6: Compound 5,7 dimethyloctahydrocoumarin 1 identified in petroleum ether cladode extract of *Opuntia ficus indica* L.

CONCLUSIONS

Opuntia ficus indica L. cladodes are found to be an excellent source of phytochemicals. Cladodes were found to be abundant in total phenolic, total flavonoid and tannin content. Cladodes also demonstrated significant antioxidant activity. Positive correlation was observed between antioxidant activity and TPC and TFC of cladode extracts of *Opuntia ficus indica* L. Our findings demonstrated that the presence of bioactive compounds like hexadecanoic acid, Limonene dioxide 2, 3, 5-Bis (trimethylsiloxy) benzoic acid, Hydrocinnamic acid, benzyldimethylsilyl ester, 5,7 dimethyloctahydrocoumarin 1 in *Opuntia ficus indica* L. cladodes were solely responsible for exhibiting antioxidant properties. These compounds can be considered promising future source of antioxidant and antimicrobial agents in *Opuntia ficus indica* L. plant. Hence, cladode extracts may confer economic and health benefits and could be utilized as nutraceuticals and food preservatives.

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Conflict of Interest. None.

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