

Antioxidant Potential of Leaves of *Opuntia monacantha* Ethanol Extract and its Fractions: An *In vitro* Study

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Abstract: The antioxidant potential of the crude ethanol, n-hexane, chloroform, ethyl acetate and remaining extracts, from the leaves of *Opuntia monacantha*, was verified using seven antioxidant assays includes: DPPH (2,2-diphenyl-1-picrylhydrazyl), phosphomolybdenum complex, reducing power (prussian blue), lipidic peroxidation (TBARS- Thiobarbituric acid reactive substances), deoxyribose oxidation and H₂O₂ scavenging. The obtained results showed that the FAE was more active in the DPPH, phosphorus-molybdenum, lipidic peroxidation, deoxyribose oxidation and H₂O₂ scavenging methods; and the FCL showed to be more active in the reduction potential method. The HCA (hierarchical cluster analysis) and PCA (principal component analysis) analysis demonstrated that DPPH is the method which requires less extract and fractions concentrations to show antioxidant potential and that deoxyribose oxidation and H₂O₂ scavenging showed a relative antioxidant activity when compared to other methods. The reduction potential and phosphomolybdenum complex showed less antioxidant potential. The cluster analysis also shows that the FAE possesses an antioxidant activity, similar to the tested standards. These results indicate that the FAE may contain promising antioxidant agents that can also be useful for the treatment of diseases caused by free radicals.

Key words: Free radicals • Antioxidant assays • PCA • HCA

INTRODUCTION

The harmful effects of free radicals have drawn the attention of scientists to the importance of antioxidants in preventing and treating diseases [1]. Hence, there is an increased interest in searching for natural products which can be used as antioxidants, preventing the oxidative damage [2, 3]. Several studies were performed with components derived from natural products, in order to unravel the components which have antioxidant properties and low cytotoxicity [4]. Natural antioxidants are important for daily consumption, while synthetic antioxidants, such as butyl hydroxyanisole (BHA), may act as carcinogenic in human beings [5].

The oxidative stress leads to serious damage to macromolecules, such as proteins and DNA. However, free radical production can be eliminated by the action of endogenous enzymes, as well as by synthetic antioxidant activity [6, 7]. Antioxidants act by several mechanisms,

including the prevention of transition metal ions-chelation catalysts, peroxidase decomposition and free radicals elimination [8].

Opuntia monacantha is popularly known as Chnutarthar from family: Cactaceae [9]. It has been used in urinary tract tumors, piles, inflammations, anemia, ulcers and enlargement of the spleen [10]. The flowers have been used for respiratory and ophthalmic complaints and fruit in gonorrhea [9]. It has a traditional use as a medicinal agent for burns, indigestion and as antipyretic agent [11]. No study concerning the phytochemical composition of its leaves has been reported so far and benzyloisoquinoline alkaloids, such as 1-(p-methoxybenzoyl)-6,7-methylenedioxyisoquinoline and 1-(hydroxy-p-methoxybenzyl)-6,7-methylenedioxyisoquinoline were isolated from this species' bark [9]. Thereby, investigate the biological activities of *Opuntia monacantha* is interesting, as the greater part of species of this genus is useful in popular medicine [10-16].

MATERIAL AND METHODS

Plant Material: Leaves from *Opuntia monacantha* were collected in April of 2010, in Taxila Rawalpindi, Pakistan and identified by Professor Dr. Masood, Botany Department, Agriculture University, Faisalabad, Pakistan. The plant *Opuntia monacantha* was compared with a voucher specimen (No. 269-1-13) deposited at the Botany Department Herbarium at University of Agriculture Faisalabad, Pakistan.

Preparation of the Crude Extract and Fractions: The collected material was oven-dried at 35°C, then milled and submitted to exhaustive extraction with ethanol, in Soxhlet equipment. After extraction, the solvent was evaporated in a rotary evaporator (Rotavapor) at 50°C and the ethanol extract, already dry, was submitted to fractioning in Soxhlet, with solvents of different levels of polarity. Then, the solvents were evaporated and the n-hexane (FH), chloroform (FCL), ethyl acetate (FAE) and remaining alcoholic (FR) fractions were obtained.

Antioxidant Activity by the DPPH Method: The quantitative antioxidant capacity to reduce the DPPH radical was measured in visible UV spectrophotometry [17]. All steps were performed in triplicate. The% of the antioxidant activity (AA%) was measured with the formula below:

$$AA\% = \frac{100 - (\text{Sample Abs} - \text{Blank Abs}) \times 100}{\text{Positive Control Abs}}$$

The values corresponding to the necessary concentration to exert 50% of the antioxidant activity (IC_{50}) were calculated in the graph, where the abscissa represents the fraction sample and the ordinate is the average of AA% of the samples in each concentration. The line equation for this graph, where $y = ax + b$, is a basis for determining the IC_{50} value.

Antioxidant Activity by the Phosphomolybdenum Complex Method: The antioxidant potential was measured by the phosphomolybdenum complex method at 695nm, in an UV-1601 Shimadzu® spectrophotometer [18]. Results were expressed as relative antioxidant activity (AAR%) of the sample, related to vitamin C, following the formula:

$$AAR\% \text{ related to vitamin C} = \frac{\text{Abs (sample)} - \text{Abs (blank)} \times 100}{\text{Abs (Vitamin C)} - \text{Abs (blank)}}$$

Antioxidant Activity by the Reduction Potential Method (Prussian Blue): The antioxidant activity was measured by Prussian blue at 700nm [19].

The results were expressed as relative antioxidant activity (AAR%) of the sample, related to vitamin C, following the formula:

$$AAR\% = \frac{\text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{Vitamin C})} - \text{Abs}_{(\text{blank})}}$$

Antioxidant Activity by the TBARS (Thiobarbituric Acid Reactive Substances) Method: Thiobarbituric acid reactive substances method was used to measure antioxidant activity at 532nm [20]. The inhibition% in lipid peroxidation was calculated by following the formula:

$$\% \text{ of inhibition} = \frac{1 - \text{Abs}_{(\text{sample})} \times 100}{\text{Abs}_{(\text{control})}}$$

Antioxidant Activity by the Thiocyanate Oxidation Method: The antioxidant activity was measured by ferric thiocyanate method at 500nm in a microplate reader [21, 22]. The antioxidant BHT (Butylated hydroxytoluene) was used as standard for these assays. The% inhibition in lipid peroxidation was calculated by following the formula:

$$AAR\% = \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{blank})} \times 100}{\text{Abs}_{(\text{control})}}$$

Antioxidant Activity by the Deoxyribose Oxidation Method: The capacity of the extracts to avoid the decomposition of deoxyribose induced by Fe^{++} and H_2O_2 was evaluated at 532nm [23]. The antioxidant vitamin C was used as standard, in a concentration of 500µg/ml.

Antioxidant Activity by the H_2O_2 Scavenging Method: The ability of the extracts to scavenge hydrogen peroxide was determined spectrophotometrically at 230nm [24]. The calculation of the scavenging activity was done by the formula:

$$\% \text{ of } H_2O_2 \text{ scavenging} = \frac{100 - \text{Abs}_{(\text{control})} - \text{Abs}_{(\text{sample})} \times 100}{\text{Abs}_{(\text{control})}}$$

Statistical Analysis: For the antioxidant assays, samples of extracts and fractions were evaluated in triplicate. Data were submitted to analysis of variance and when the effects of the treatments were significant, related to the control, averages were compared by Tukey test.

All results were analyzed considering a significance level $\alpha = 5\%$. The obtained results were expressed as mean \pm standard deviation [18-23].

Chemometric Treatments: Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) data was based on biological activity information. For each activity, two chemometric matrices were evaluated: first, the % antioxidant values, IC and MIC were interpreted to delineate the general behavior of each extract and fractions against the antioxidant methods, bacterial and cellular lineages. Then, data of each activity were evaluated to specify the antioxidant, antimicrobial and cytotoxic potentials, against the antioxidant methods, bacterial and cellular lineages. Hence, a PCA was performed through pre-processing of self-adjusted means with varied scales, which attribute to the same variant charge. For HCA, matrices were also auto-adjusted and Euclidean distances among extracts and fractions were calculated. The incremental linker method (an approach of the sum of squares to calculate intercluster distances) was applied to generate clusters [25, 26].

RESULTS

The extract and fractions originated from *O. monacantha* showed an antioxidant activity which was dependent of the used method. The DPPH showed that the FAE possesses an antioxidant action which is similar to vitamin C, with an IC_{50} value of $4.2\mu\text{g/ml}$ and the FR to the rutin standard ($IC_{50}=7.7\mu\text{g/ml}$). Although the EEB and FCL did not show a similar activity to standards, only 15.7 and $15.5\mu\text{g/ml}$ were necessary to achieve the IC_{50} (Figure 1A). No extract or fraction showed antioxidant activity similar to the standards vitamin C and rutin using the phosphorus-molybdenum complex method and a small activity was observed for the FAE (36.5% of activity) (Figure 1B). The Reduction Potential method (Prussian blue) showed that FCL had a similar activity to the standard rutin (65.1% of activity). EEB presented an antioxidant activity of 81.5%, considered more active when compared to the fractions (Figure 1C). For the lipid peroxidation assay, FH (74%), FCL (75%) and FAE (75.6%) showed an inhibition activity of more than 50%. However, these values were not superior to the standard BHT (84.3%) (Figure 1D).

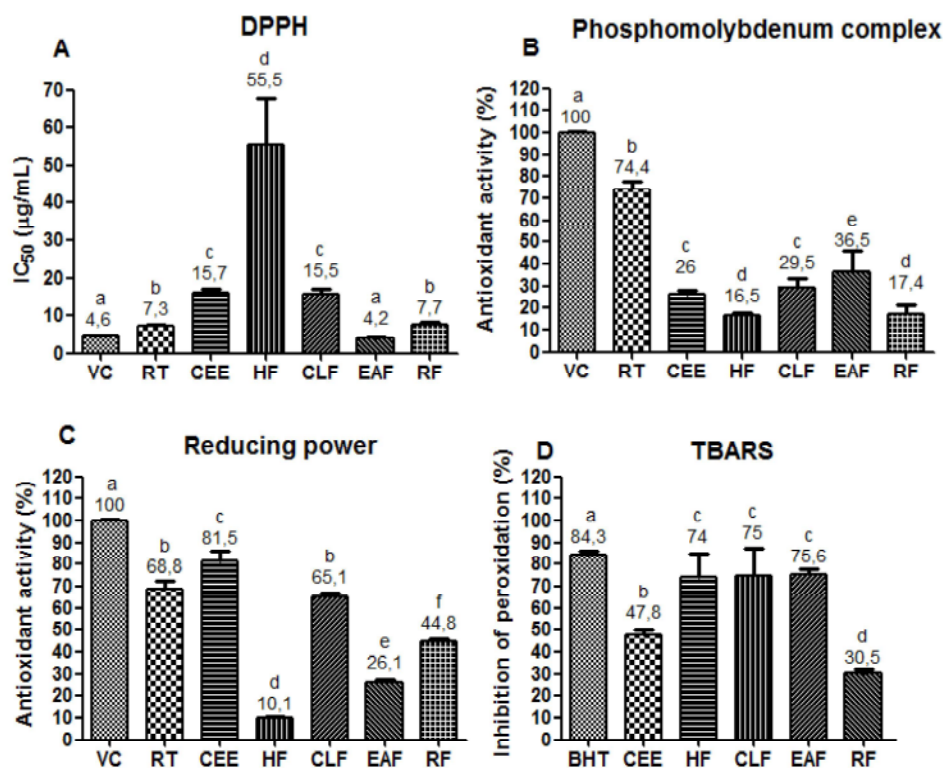


Fig. 1: Antioxidant activity of the crude ethanol extract (EEB), n-hexane (FH), chloroform (FCL), ethyl acetate (FAE) and remaining fractions (FR) of *O. monacantha*, by A: DPPH, B: Phosphomolybdenum complex, C: Reducing Power and D: TBARS. Means followed by the same letter do not differ each other by the Tukey test, $p < 0.005$.

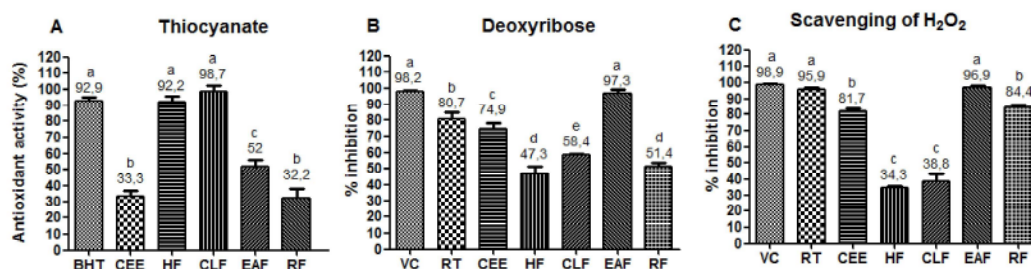


Fig. 2: Antioxidant activity of the crude ethanol extract (EEB), n-hexane (FH), chloroform (FCL), ethyl acetate (FAE) and remaining (FR) fractions of *O. monacantha*, by the methods A: thiocyanate inhibition, B: deoxyribose inhibition and C: hydrogen peroxide screening. Averages followed by the same letter do not differ each other by the Tukey test, $p < 0.005$.

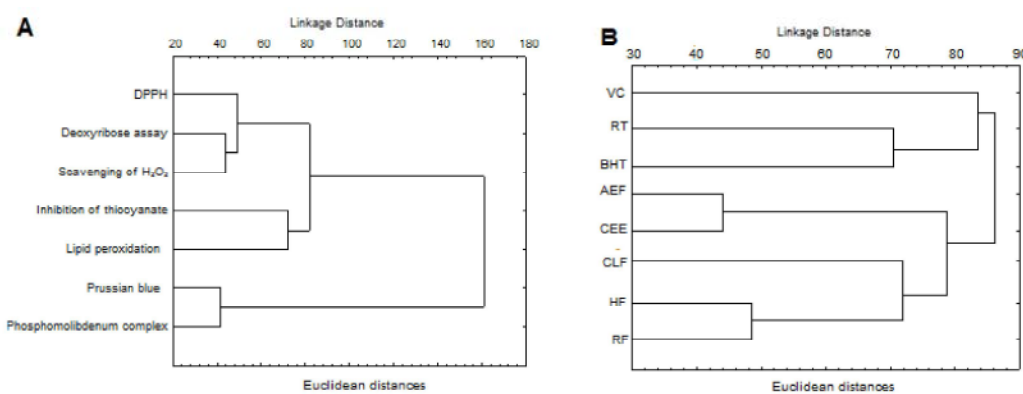


Fig. 3: Built Cluster, from antioxidant activity data, for the tested antioxidant methods (A) and standards Vitamin C (VC), rutin (RT) and BHT, ethanol crude extract (EEB), n-hexane (FH), chloroform (FCL), ethyl acetate (FAE) and remaining (FR) fractions (B) of *O. monacantha*. The cluster was performed with Euclidean distances, using an incremental linkage method to generate clusters. Branches represent stronger activity (upper portion, near to DPPH and standard vitamin C methods), intermediate and weak activities.

Similar to the lipid peroxidation inhibition method, FAE presented an antioxidant activity of 52% by the thiocyanate inhibition method. FH (92.2%) and FCL (98.7%) had similar activities to BHT (92.9%) (Figure 2A) in the thiocyanate inhibition method. For the deoxyribose method, only FH did not show an inhibition superior to 50%. Only FAE showed an activity of 97.3% and its activity was considered similar to vitamin C (98.2%). EEB (74.9%), FCL (58.4%) and FR (51.4%) showed an activity superior to 50% as well (Figure 2B). FAE showed an antioxidant activity similar to vitamin C and rutin, with an inhibition of 96.9% of the H₂O₂ radical (Figure 2C). A low activity was verified for FH and FCL, demonstrating that these fractions are not considered active by this method. EEB and FR showed activity between 81.5% and 84.4%.

A hierarchical cluster analysis (HCA) was performed for all the experimental data, taking the Euclidean distance as metric and the complete linkage method, as an amalgamation method. The obtained results were divided

in three distinct groups, which correspond to three antioxidant levels of extracts and fractions. As demonstrated in Figure 3, the DPPH method was characterized as the method which detects a higher antioxidant action, when compared to the others, followed by the deoxyribose reduction and H₂O₂ scan. The thiocyanate inhibition method and lipid peroxidation show as medium activity methods and the reduction potential and phosphorus-molybdenum complex demonstrated to be less effective for the EEB and fractions of *O. monacantha* (Figure 3A).

When the antioxidant potential among EEB and fractions was compared, a grouping indicated that FAE and EEB presented a higher antioxidant potential by the tested methods. FH and FCL would be considered fractions with an intermediate activity and FR the fraction which showed smaller activity (Figure 3B).

Data obtained in the antioxidant activity evaluation were used to perform a graph, where the loadings of the principal component analysis (PCA) correspond to

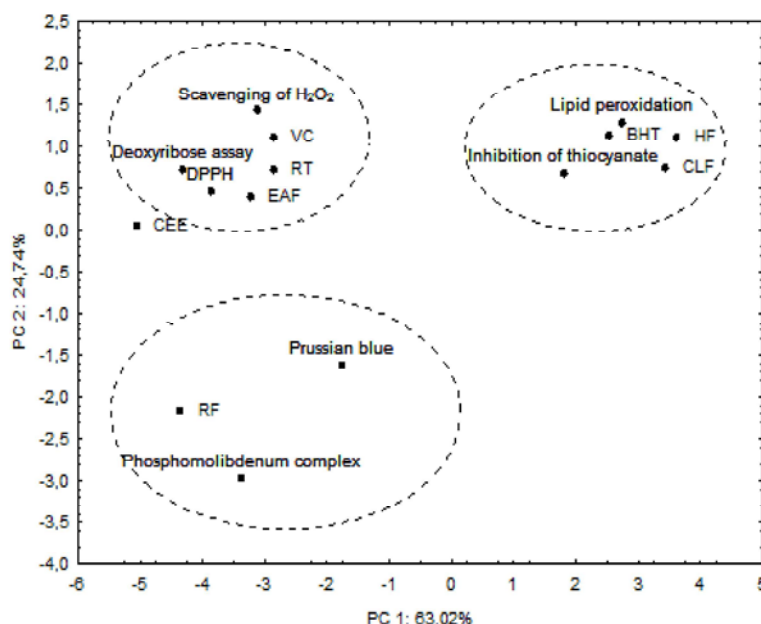


Fig. 4: PCA which represents the evaluated parameters to the antioxidant activity with data parcels for the standard and extracts. The parcels show 63.02 and 24.74% of the total variance explained in PC1 and PC2, respectively.

antioxidant activity. The PC1 axis corresponds to 63.02% of variance, while the PC2 axis represents 24.74% and these axis explain the antioxidant effect under EEB and tested fractions.

The extracts and fractions designated to elevated values over the PC2 axis have a pronounced antioxidant activity in FAE, EEB and standards vitamin C and rutin, indicating that these fractions and standard showed a higher activity for DPPH, deoxyribose inhibition and H₂O₂ scan methods. The methods which are closer to negative values for PC₂ and PC1 indicate a less antioxidant potential for FR and prussian blue and phosphomolybden complex. The BHT, FH and FCL standards, localized on the positive values of PC1 and PC2, indicates high activity for the methods of lipid peroxidation (TBARS) and thiocyanate inhibition (Figure 4).

DISCUSSION

The analysis of the crude ethanol extract and fractions obtained from leaves of *O. monacantha* indicated that the activity is concentrated in the crude extract and in the ethyl acetate fraction. The main reason for a less antioxidant activity of the n-hexane and chloroform fractions against the DPPH, reduction potential, phosphomolybden and H₂O₂ radical potential may be correlated to solubility and stability of the

compounds responsible for antioxidant activity which may be a polar compound. These pharmacokinetic properties are closely related to the pharmacological efficacy, as the antioxidant efficacy depends on the capacity of the compounds to penetrate the cellular membrane [27].

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