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Variation In Antioxidant Activity And Phyto-Constituents In Different Parts Of Pyracantha Crenulata Collected From Middle Hill Climatic Condition Of Western Himalayas

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ABSTRACT

The variation in antioxidant activity and phyto-constituents of aqueous and alcoholic extracts of different plant parts (i.e. Leaves, roots, stems, and fruits) of Pyracantha crenulata was determined and the correlation between phyto-constituents and antioxidant activities was also explored. The result revealed that the alcoholic extract of leaves exhibited maximum antioxidant activity with the lowest IC_{50}/EC_{50} value by ABTS (0.029 mg/mL), DPPH (0.047 mg/mL), and PFRAP (0.025 mg/mL) followed by the roots of the plant. A similar pattern was observed in their phyto-constituents, i.e., the leaves exhibited maximum phenolic (127.49 mg/g), flavonoid (23.20 mg/g), and tannin contents (152.32 mg/g) followed by the roots. Similarly, the aqueous extract of P. crenulata leaves showed the highest antioxidant activity and phyto-constituents followed by roots. The correlation analysis revealed that the phyto-constituents exhibited a significant negative correlation with the IC_{50}/EC_{50} value and the IC_{50} value of ABTS assay had a significantly positive correlation with the IC_{50} and EC_{50} value of DPPH and PFRAP assay, respectively. The study revealed that the alcoholic extract of the plants possesses a significantly higher antioxidant activity and

phyto-constituents than the aqueous extract. Further, this difference could attribute to an uneven distribution of phytochemical contents in the different parts of the plant and, therefore varying its antioxidant activity.

Keywords Antioxidant assay, Correlation, Constituents, IC_{50}value, Plant parts

1. Introduction

An antioxidant is a chemical that prevents other molecules from oxidation. Oxidation is a chemical reaction in which electrons from a compound are exchanged to an oxidizing agent. These oxidation mechanisms can produce free radicals that could initiate chain reactions, resulting in cell death and damage (Alam, 2013). By scavenging free radicals and suppressing oxidation reactions, antioxidants can interrupt chain reactions. To counteract the negative effects of oxidants, the human body contains an inherent defense mechanism consisting of endogenous antioxidants(Simpson, 2015).Antioxidants can decrease oxidative stress in the body by scavenging reactive oxygen species (ROS) to either prevent or repair the damage.Under oxidative stress, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) provide the first line of defense against free radical damage (Verma, 2013; Anderson, 2004). The phenolic compounds such as glutathione (GSH), ascorbic acid (vitamin C), and α-tocopherol (vitamin E) are non-enzymatic antioxidants that provide defense by changing oxidants to non-radical end products or transferring radicals to zones where their effects are less harmful (Simpson, 2015). Vitamin A, C, and E, rich vegetables and fruits, provide a healthy defense against free radical production while increasing the number of cell receptors available for antioxidant enzyme activation (Parletta,2013). Antioxidant polyphenols in the diet protect against a variety of degenerative diseases, including diabetes, cancer, and cardiovascular disease, and help to prevent oxidative stress (Scalbert, 2005; Nijveldt,2001).

Pyracantha crenulata (Roxb. ex D. Don) M. Roem. or Crataegus crenulata Roxb belongs to family Rosaceae (Negi, 2018). The genus Pyracantha is an evergreen, thorny shrub, and 10 species of the genus known as ‘firethor’ worldwide due to its dense poisonous thorns and it is generally used for fencing purposes (Javad, 2020, Gu C, 2003). Pyracantha crenulata is an evergreen, deciduous, perennial, and thorny shrub, commonly called Himalayan Firethorn or Indian hawthorn or Ghingharu (Singh, 2012). The dense bushy shrub is native to the Mediterranean to cool, subtropical climates of Southeast Asia (Javad, 2020; Wang, 2006, Liu, 2010). In India, the plant is distributed in the temperate Himalayas at 900 m to 2400 m altitude (Kunwar, 2010, Javad, 2020) and is abundantly found in barren, rocky, and dry grasslands. It is a thick shrub to small tree with short spiny branches and erect, sharp angle stem; grow up to 3-5 m high. Dark green leaves are simple, sessile, lanceolate to ovate with short petiolate,

stipulate, alternate, and pubescent, up to 5 cm long. The plant has a terminal inflorescence with many flowers, and the flowers are white to creamy white pentamerous and lanceolate. The flowers have acute, triangular, persistent sepals and ovate to sub-orbicular petals with short filament (2-4 mm). The fruits are red-to-orange, small fleshy pome-like a tiny apples with many irregular seeds (Csurhes, 2016; Javad, 2020; Gu C, 2003). The different parts of the plant contain many bioactive molecules viz., vitexin4 rhamnoside, vitexin, leucocynidine, leucoanthocyanidin, flavanoids, flavonol, kaempferol, glycoside, quercetin, beta-sitosterol, and oligomeric saponins, etc. (Negi, 2018).

It holds medicinal utilization in treating cardiac failure, paroxysmal tachycardia, myocardial weakness, hypertension, and arteriosclerosis. Besides, the fruits manifest antispasmodic, diuretic, sedative and vasodilatation properties. The analysis of the proximate, elemental composition and phytochemical investigations will givea prelude to the therapeutic potential and other possible applications of the plant (Karthika, 2018). The present study aimed to evaluate the variation in the in-vitro antioxidant activity and phyto-constituents of aqueous and alcoholic extracts of different plant parts of Pyracantha crenulata. Further, the correlation analysis was established between the antioxidant activity and phyto-constituents.

2. Materials And Methods

2.1. Extraction of different plant parts

Different plant parts, viz. leaves, roots, stems, and fruits of Ghingaru (Pyracantha crenulata) collected from Pithoragarh, Uttarakhand (India), dehydrated, powdered, and stored carefully in a tightly-closed container for further studies. Further, the plant was authenticated by the Botanical Survey of India (BSI), Dehradun (Accession No. 502). The dried powder of different plant parts, viz. leaves, roots, stems, and fruits (50 gm), were successively extracted by cold maceration process, three times with occasional shaking 500 ml of water and ethanol for aqueous and alcoholic extracts, respectively. Then the collected filtrates were concentrated to obtain semisolid residue in-vacuo using a rotary vacuum evaporator.

2.2. Chemicals

Reagents purchased from Sigma Chemicals USA were of analytical grade. Ascorbic acid, DPPH (1,1-diphenyl-2-picrylhydrazyl), tannic acid, ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), sodium carbonate, potassium persulfate ($K_2S_2O_8$), potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), potassium acetate (CH_3COOK), and Folin-Ciocalteau reagent.

2.3. In-vitro antioxidant activity in different parts of the plant

For the estimation of antioxidant activity the concentrated aqueous and alcoholic extracts of different plant parts were dissolved in their respective solvents for preparing a solution having a concentration of 100 mg/10 mL. For antioxidant assays, the concentrations of the above solution were diluted accordingly. The reference standard for the antioxidant assays in the present study was ascorbic acid (0.1 mg/mL). Different antioxidant assays were used for the evaluation of antioxidant activity and the analysis was carried out in replication (n=3).

2.3.1. ABTS free radical scavenging assay

The assay is based on the principle of scavenging of blue-green radical cation ($ABTS^{\bullet+}$), which is formed by the treatment of 7 mM ABTS (2,2-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid)) with sodium/potassium persulphate. The antioxidant potential was evaluated as per the described method (Re, 1999). The antioxidant capacity of plant samples was observed by taking different concentrations of the sample (00-50 µL), diluted with distilled water up to 1 mL, then 1 mL of ABTS solution added into it. All the samples were kept in the dark for 5-7 min. and then immediately noted the absorbance at 734 nm. A decrease in the absorbance was observed due to the reduction of ABTS radical by the hydrogen donating antioxidant, which causes the change in the color of blue ABTS radical to colorless ABTS (Guglani, 2020).

The % free radical scavenging activity (FRSA) was determined by the following formula:

$$\text{FRSA (\%)} = \frac{\text{Absorbance of control sample} - \text{Absorbance of the test sample)}}{\text{The absorbance of control sample}} \times 100$$

The inhibition concentration value (IC_{50}) was calculated as:

$$\text{IC50 value (mg/mL)} = \frac{\text{Concentration of test sample}}{\text{50\% nearest FRSA}} \times 50$$

2.3.2. DPPH free radical scavenging assay

The assay is based on the scavenging of violet-colored DPPH free radical (1,1-diphenyl-2-picryl-hydrazyl) by the hydrogen donating antioxidants. The antioxidant potential was evaluated according to the described method (Kedare, 2011). For the determination of the antioxidant capacity of plant samples by the DPPH assay firstly, 0.135 mM of DPPH methanolic solution was prepared. After that, different concentration of the sample (00-50µL) was taken and diluted with distilled water up to 1 mL. Now, 2 mL

of DPPH methanolic solution was added to each sample tubes. All the samples were kept under dark for 40 min., and then immediately noted the absorbance at 517 nm against a blank (methanol). A decrease in the absorbance was observed due to the reduction of DPPH radical by the hydrogen donating antioxidant, which causes the change in the color of violet DPPH free radical to pale yellow or colorless DPPH. The percentage free radical scavenging activity (FRSA) and IC_{50} value were determined by the same formulas as stated above (Chandra, 2019;Guglani, 2020).

2.3.3. The PFRAP (Potassium ferricyanide reducing power) Assay

The assay is based on the principle of reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). In this assay, the antioxidant, this has a reducing ability to convert the potassium ferricyanide into potassium ferrocyanide. Further, generated a ferric ferrocyanide complex, an intense prussian blue colored complex by reacting with the ferric trichloride ($FeCl_3$), and shows the absorbance maxima at 700 nm (Maruthamuthu, 2016). For the evaluation of antioxidant capacity, different aliquots of plant samples were taken and mixed with 2.5 mL of phosphate buffer (6.6pH). Then added 2.5 mL of 1% w/v solution of potassium ferricyanide ($K_3Fe(CN)_6$) and incubated the solution for 20 min., at 50°C. After incubation, added 2.5 mL of 10% v/v trichloroacetic acid solution in each tube and centrifuged the mixture for 10 min. After the centrifugation process, taken the 2.5 ml of supernatant from the mixture and diluted it with an equal amount of distilled water, and finally added the 0.5 mL of 0.1 % w/v $Fecl_3$ solution into it. Vertex the resultant mixture and immediately measure the absorbance at 700 nm. EC_{50} value (concentration of the sample to get 0.5 absorbances) was calculated to measure the reducing ability of samples (Guglani, 2020).

2.4. Determination of phyto-constituent

2.4.1. Total phenolic contents

The total phenolic contents (TPC) of the samples were evaluated by the Folin- Ciocalteu colorimetry method. According to the described method (Shoib, 2015), take 1 mL of the extract solution and evaporate to dryness. After that, it dissolved the residue into the known volume of distilled water and taken the sample from the above solution, and further diluted it with the 3 mL of water. Added 0.5 mL of Folin-ciocalteu reagent into each tube and kept aside for 3 min. After, that 2 mL of 20 % sodium carbonates (Na_2CO_3) solution was added and mixed thoroughly. Placed the samples into the boiling water bath for 1 min and noted the absorbance at 650 nm. Total phenolic contents were calculated by Catechol standard calibration curve and the curve was prepared by taking a different concentration of

Catechol (50-300 µg/ml). The data were expressed in mg/g of catechol on a dry weight basis (Guglani, 2020; Dhull, 2016).

2.4.2. Flavonoid contents

The flavonoid contents (TFC) of the samples were measured by the aluminum chloride colorimetric method (Chang, 2002). In this method, the extract samples (100 µL) were taken in the test tubes and then added the 400 µL of 80% ethanol after that subsequently added the 1.5 mL of 95% ethanol. In the resultant solution, added the 100 µL of aluminum chloride solution followed by the 100 µL of potassium acetate solution. Then thoroughly mixed the samples by cyclo-mixture at 1000 rpm and incubated at room temperature for 30 min. After that, immediately measured the absorbance at 415 nm versus the blank solution (containing the entire reagent except for aluminum chloride). Total flavonoid contents were calculated by Quercetin standard calibration curve and the curve was prepared by taking different concentrations of Quercetin (50-300 µg/mL). The data were expressed in mg/g of Quercetin on a dry weight basis (Guglani, 2020; Pandey, 2019).

2.4.3. Tannin contents

The total tannin contents (TTC) of the samples were measured by the Folin-Denis colorimetric method (Sadasivam, 2007). In this method, firstly taken about 100 µL of the leaves to extract samples into the 50 mL volumetric flask, which contains 30 mL of distilled water. After that 2.5 mL of Folin-denis reagent was added followed by the 5 mL of 35 % sodium carbonate solution were added. Finally, the volume was makeup with the distilled water up to the mark. After that, the resultant solutions were mixed thoroughly and kept at 10-20°C for 30 min. The absorbance of the samples was then measured at 700 nm against the black solution. Total tannin contents were calculated by Tannic acid standard calibration curve and the curve was prepared by taking a different concentration of Tannic acid (50-300 µg/mL). The data were expressed in mg/g of Tannic acid on a dry weight basis (Guglani, 2020; Pandey, 2019).

2.5. Statistical analysis

The results were quantified by Mean ± Standard Deviation (SD) (n=3). Using SPSS 16.0 Software, the collected data were analyzed using one-way analysis of variance (ANOVA) and Duncan's test at the 0.05 probability level. The difference between the means ($P<0.05$) was considered to be significant.

3. Results And Discussion

3.1. In-vitro antioxidant activity in different parts of the plant

The in-vitro antioxidant activity was assessed by three different assays viz. ABTS, DPPH, and PFRAP assay. The capacity of the extracts to inhibit the ABTS, DPPH radicals, and reducing power was expressed by estimating IC_{50} value (mg/mL) for both aqueous and alcoholic extracts of different parts of the plant viz. leaves, stems, fruit, and roots (Figure 1.).

The inhibition concentration (IC_{50}) was estimated in the study; minimum IC_{50} value means maximum anti-oxidant activity. The ascorbic acid was used as a reference standard, and the IC_{50} value was 0.00449 mg/mL. In an aqueous extract of P. crenulata leaves; a minimum IC_{50}value was exhibited by leaves (0.034 mg/mL) followed by the root (0.0563 mg/mL). While the fruits extract shows the least antioxidant activity. Similarly, the alcoholic extract of P. crenulata leaves showed the highest antioxidant activity (0.0296 mg/mL) followed by roots (0.036 mg/mL), and minimum antioxidant activity was exhibited by the fruits. The alcoholic extract showed better antioxidant activity followed by the aqueous extract.

In the aqueous extract of P. crenulata leaves; a minimum IC_{50} value was exhibited by leaves (0.051 mg/mL) followed by the root (0.077 mg/mL). While the extract of the fruit shows the least antioxidant activity. Similarly, the alcoholic extract of P. crenulata leaves showed the highest antioxidant activity (0.0473 mg/mL) followed by roots (0.0563 mg/mL), and minimum antioxidant activity was exhibited by the fruits. The alcoholic extract showed better antioxidant activity followed by the aqueous extract.

The reducing power (EC_{50}) in aqueous extracts of P. crenulata ranged from 0.063 to 0.565 mg/mL. The results revealed that the maximum reducing power observed in leaves extract (0.063 mg/mL) followed by root extract (0.113 mg/mL) and minimum activity displayed by the fruits. In alcoholic extract, leaves extract displayed highest reducing power (0.025 mg/mL) followed by in roots extract (0.054 mg/mL). The alcoholic extract showed better antioxidant activity followed by the aqueous extract.

The present study compared the antioxidant potential of aqueous and alcoholic extracts of the different parts against different free radicals. The antioxidant activity was estimated by inhibition concentration (IC_{50}); the leaves displayed better antioxidant activity followed by the other parts of the plant.

3.2. Determination of phyto-constituent

The phyto-constituents viz. total phenolic, flavonoid, and tannin contents were assessed in aqueous and alcoholic extracts of different parts of the plant viz. leaves, stems, fruit, and roots (Figure 2.)In the aqueous extract, P. crenulata, phenolic contents varied from 20.81 to 76.436 mg CE/g d.w. Maximum phenolic contents (76.436 mg/g) were found in the aqueous extract of leaves followed by the root (52.476 mg/g). While least concentration of phenolics was recorded from fruits extract. The phenolic contents in the alcoholic extract were ranged from 39.01 to 127.496 mg CE/g d.w. In the alcoholic extract, the highest phenolic contents were found in leaves (127.496 mg/g) followed by the roots (88.667 mg/g). Maximum phenolic contents were found in alcoholic extracts of different parts as compared to the aqueous extracts.

In aqueous extract P. crenulata, flavonoid contents varied from 2.52 to 10.63 mg QE/g d.w. Maximum flavonoid contents (10.63 mg/g) found in aqueous extract of leaves followed by the root (7.84 mg/g). While least concentration of flavonoid was recorded from fruits extract. The flavonoid contents in the alcoholic extract were ranged from 4.15 to 23.20 mg QE/g d.w. In the alcoholic extract, the highest flavonoid contents were found in leaves (23.20 mg/g) followed by the roots (15.14 mg/g). Maximum flavonoid contents were found in alcoholic extracts of different parts as compared to the aqueous extracts.

In aqueous extract P. crenulata, tannin contents varied from 27.36 to 134.25 mg TAE/g d.w. Maximum tannin contents (134.25 mg/g) found in aqueous extract of leaves followed by the root (75.23 mg/g). While least concentration of tannin was recorded from fruits extract. The tannin contents in the alcoholic extract were ranged from 55.46 to 152.32 mg TAE/g d.w. In the alcoholic extract, the highest tannin contents were found in leaves (152.32 mg/g) followed by the roots (113.95 mg/g). Maximum tannin contents were found in alcoholic extracts of different parts as compared to the aqueous extracts. The leaves showed higher total phenolics, flavonoids, and tannin contents followed by other parts. The results revealed the variation of IC_{50}, EC_{50} values, total phenolic, flavonoids, and tannin contents that may be attributed to a different part of the plant used for the study. Further, this difference could attribute to an uneven distribution of phytochemical contents in the different parts of the plant and therefore, varying its antioxidant activity.

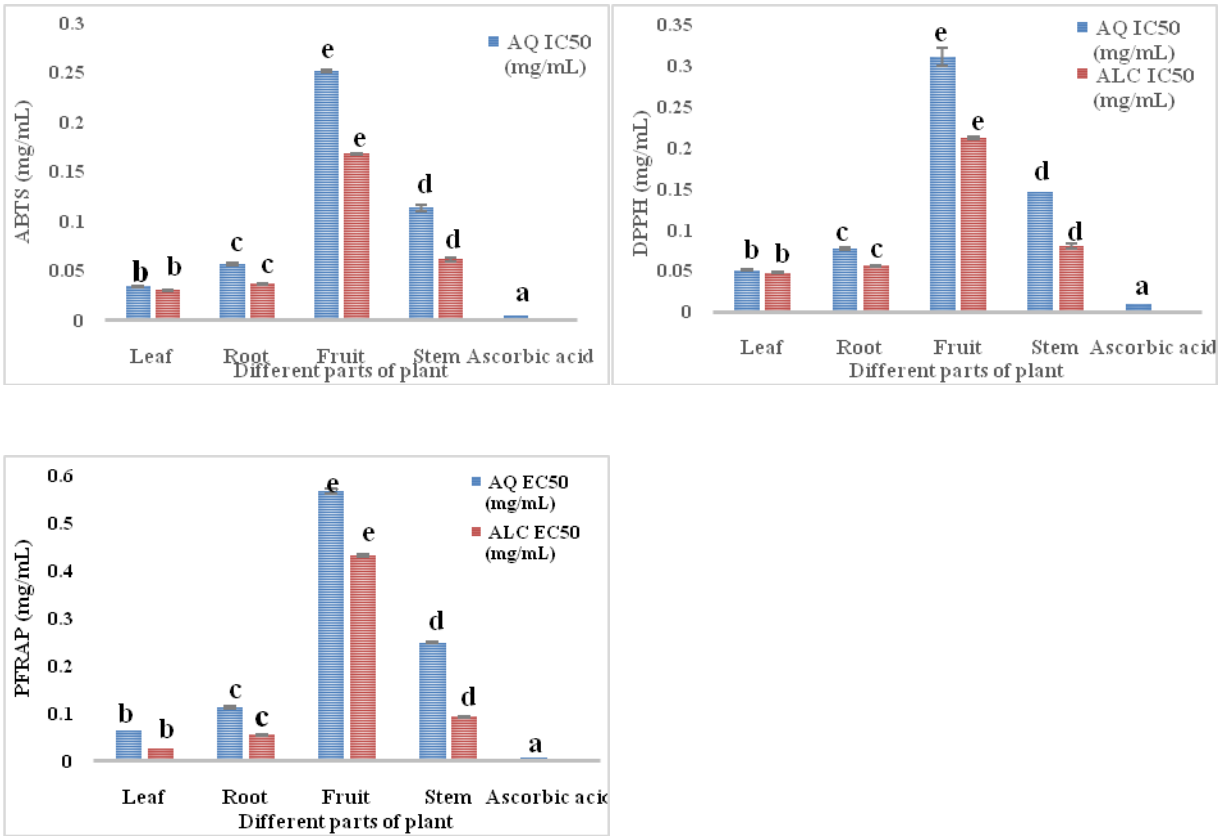

Figure 1. Antioxidant activity in the aqueous and alcoholic extracts of different plant parts of P. Crenulata by ABTS Assay (a), DPPH Assay (b), and PFRAP Assay. Different alphabets represent the significant difference (P <0.05) according to Duncan's and LSD tests.

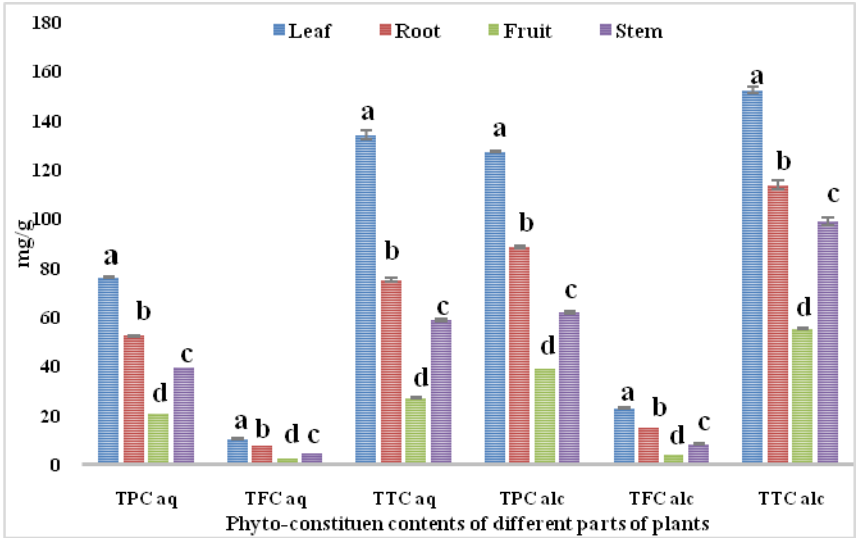

Figure 2. The phyto-constituents contents in the aqueous and alcoholic extracts of different plant parts of P. crenulata. Different alphabets represent the significant difference (P <0.05) according to Duncan's and LSD tests.

3.3. Correlation Analysis

The Pearson's correlation analysis of phyto-constituentssuch as total phenolic, flavonoids, and tannin contents with the antioxidant potential of different parts of P. crenulata are represented in Table 1.The values of the correlation coefficient showed that the phyto-constituents exhibited a significant negative correlation with the IC_{50}/EC_{50} value of different antioxidant methods such as the ABTS, DPPH, and PFRAP assay. The results also revealed that the IC_{50} value of the ABTS assay had a significantly positive correlation with the IC_{50} and EC_{50} value of the DPPH and PFRAP assay, respectively.

Therefore, the data reflects that the higher the contents of phyto-constituents, the lower the IC_{50}/EC_{50} value, and the higher will be the antioxidant potential. The data suggested that the phenolic, tannin, and flavonoid constituents are the significant contributors to the antioxidant activity of the plant extracts. The findings also revealed that the IC_{50} value of the ABTS assay of all the samples had a significant positive correlation with their IC_{50} value of DPPH assay and an EC_{50} value of the FRAP method. The current study data was in accordance with the findings of previous workers, which reported the correlation of phyto-constituents with the antioxidant potential (Fidrianny, 2015; Tamuly, 2014).

Table 1. Pearson's correlation analysis of phyto-constituents with antioxidant activities of different plant parts of P. crenulata

Antioxidant parameters	Correlation coefficient (r)					
	IC_{50}ABTS assay	**IC_{50}DPPH assay**	**EC_{50}PFRAP assay**	**TPC**	**FC**	**TC**
IC_{50} ABTS assay	1	1.00**	0.993**	-0.791*	-0.730*	-0.875**
IC_{50} DPPH assay		1	0.993**	-0.785*	-0.723*	-0.872**
EC_{50} PFRAP assay			1	-0.803*	-0.750*	-0.879**

TPC	1	0.990**	0.946**
FC		1	0.896**
TC			1

** Indicates correlation significance at P < 0.05 (2-tailed) and *Indicates correlation significance at P < 0.01 and. TPC- total phenolic content, FC-flavonoid contens, and TC-tannin contents.

4. Conclusion

The study concluded that the alcoholic extracts of leaves of Pyracantha crenulata exhibited significantly higher antioxidant activity and phyto-constituents than aqueous extract. Further, the leaves showed significantly maximum antioxidant activity and phyto-constituents followed by the roots, and the least antioxidant activity and phyto-constituents were exhibited by the fruits. The phyto-constituents viz. total phenolic, flavonoids, and tannin contents of different parts of Pyracantha crenulata exhibited significant negative correlation with its antioxidant activity. Therefore, the study reflects that the phenolic, tannin, and flavonoid constituents are the significant contributors to the antioxidant activity. Consequently, the findings provide substantial evidence that the leaves of Pyracantha crenulata can be valued as a source of effective natural antioxidants with consequent health benefits.

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