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The antioxidant activity, total phenolic and monomeric anthocyanin content of *Gymnema sylvestre* leaf aqueous extract

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Abstract

The *Gymnema sylvestre* is a widely recognized medicinal plant, particularly for its effectiveness in treating diabetes. Aim of this study is to measure the antioxidant property, polyphenol and anthocyanin content of *G. sylvestre* aqueous extract using various in-vitro techniques. The aqueous extract was prepared and total phenolic compounds (TPC), DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical scavenging ability, total antioxidant activity using ferric reducing antioxidant power (FRAP), ferric reducing power and monomeric anthocyanin content were estimated. Correlation analyses were performed for TPC and anthocyanin against the antioxidant activity. The TPC was 4.55±0.34 mg g^{-1} of fresh leaf weight (FLW) and the IC50 value for DPPH radical scavenging ability was 2.40 mg mL^{-1} of leaf extract concentration. The percentage of inhibition strongly correlated (R^2 = 0.99) with the concentration of the leaf extract. The total antioxidant power of the leaf extract was 21.06±1.74 µmol g^{-1} of FLW.

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The ferric-reducing power showed a positive correlation with the leaf concentration (R^2 = 0.99). The anthocyanin content of the leaf extract was 167.84±1.45 mg per 100 g of leaf. The TPC and anthocyanin exhibited strong positive correlations with total antioxidants and DPPH, but a weak correlation with reducing power. The antioxidant properties of leaf extract could be ascribed to the presence of TPC and anthocyanin as it showed a strong correlation with antioxidant activity. This is the first study that reported the anthocyanin content of G. *sylvestre* leaf extract. This study found G. *sylvestre* leaf water extract has antioxidant properties and contains a significant amount of TPC and monomeric anthocyanin.

Keywords: *Gymnema sylvestre;* Antioxidant activity; DPPH radical scavenging ability; Total antioxidant activity; Total phenolic content.

Introduction

Gymnema sylvestre is a woody type vine used in folk, ayurvedic and homoeopathic medicine as it has several medicinal values. This plant belongs to the family Asclepiadaceae or the "milkweed" family (Saneja *et al.,* 2010). *G. sylvestre* has been prescribed for various complications, including diabetes, obesity and liver diseases in folklore and traditional system of medicine. Many beneficial characteristics of *G. sylvestre* were reported in the medical field (Jayachitra, 2015). Triterpene saponins are major constituents of this plant which belongs to oleanane and dammarene classes. Gymnemic acids and gymnemasaponins belong to oleanane-type saponins and gymnemasides belongs to dammarene saponins (Khramov *et al.*, 2008). The main secondary metabolites of *Gymnema* are acidic glycosides. Of that, gymnemic acids A to D are the main secondary metabolites which found in all parts of the plant. These substances have the potential to act against reactive species.

Reactive oxygen (ROS) and nitrogen (RNS) species are free radicals having a short life due to their instability, thus highly reactive with biological substances. These free radical species alter the structural and functional properties of biological molecules and result in "Oxidative Stress" (Karigidi *et al.*, 2019). These free radicals increase the rate of ageing and loss of cell function due to the degradation of macromolecules like DNA, RNA, lipid and protein (Kotásková *et al.,* 2016). Our body has natural mechanisms to control oxidative damage.

However, it needs additional support from other sources like food or medicine to fight against them when it surpasses the threshold level.

Antioxidants are chemical substances which have the ability to neutralize or stabilize the functions of free radicals (Kostuyk and Potapovich, 2009). Plants derived antioxidants are widely used as antioxidants which have various physiochemical properties (Satué-Gracia *et al.,* 1997). To mitigate the negative impact of free radicals and supplement cellular defence mechanisms, synthetic antioxidants like butylated hydroxyanisole, butylated hydroxytoluene, and tertiary butyl hydroquinone are frequently employed in the food industry (Jayaprakasha *et al.*, 2000). However, the use of synthetic antioxidants as a feed additive is restricted or prohibited in many countries due to their toxic effect. Therefore, researchers pay high attention in recent times to find alternative antioxidant derived from plants (Meghana *et al.*, 2007).

Around 8,000 polyphenolic compounds are identified from different plant sources (Pandey and Rizvi, 2009). Phenolic acids, flavonoids, stilbenes and lignans are main polyphenols in plants (Spencer *et al.,* 2008). Polyphenols have garnered significant attention for their biological activities, particularly their capacity to quench free radicals and inhibit lipid peroxidation, making them vital antioxidants (Djeridane *et al.,* 2006). Phenolics exhibit antioxidant activity through their redox ability, which enable them to function as metal chelating agents, electron donors, and reducing agents (Rice-Evans *et al.*, 1995). Studies have suggested that polyphenols could safeguard cellular components against oxidative damage and associated degenerative illnesses (Luqman and Rizvi, 2006). Intake of polyphenols minimise the changes of coronary heart diseases, reduction of several types of tumours or their growth (Yang *et al.,* 2001), decreases the risk of diabetes (Rizvi and Zaid, 2001), oxidative stress and damage to brain macromolecules in neurodegenerative diseases (Letenneur *et al.,* 2007). Besides polyphenols, anthocyanin also fights against oxidative damages. Anthocyanin belongs to class of molecules named flavonoids and it is synthesized through phenylpropanoid pathway. Anthocyanin could minimise damage to DNA, anti-inflammatory activity, inhibition of enzyme, etc (Lazze *et al.,* 2003). The ability of anthocyanin to reduce cancer cell proliferation and inhibit tumour formation is strongly related to its antioxidant property (Lila, 2004).

Previous studies have shown that the alcoholic or water-alcohol extract of *G. sylvestre* leaf has significant antioxidant properties (Gunasekaran *et al.,* 2019, Abhiram *et al.,* 2023). But in a real-world application, this plant is being consumed as a food or medicine where the antioxidant activity depends on the aqueous media. However, only very limited studies reported the antioxidant property of *G. sylvestre* leaf in the aqueous medium (Kang *et al.,* 2012, Arunachalam *et al.,* 2014) and no studies were available for this plant grown under Sri Lankan climatic conditions. Nevertheless, the monomeric anthocyanin content of *G. sylvestre* has not been reported elsewhere. Therefore, this study was designed to explore the potential antioxidant activity of *G. sylvestre*in aqueous media and estimate the relationship between the antioxidant activity and phenolic compounds present in the extract.

Materials and methods

Leaf collection and extract preparation

Fresh and mature leaves of *G. Sylvestre* were gathered six times from Jaffna, Sri Lanka. For every assay, 300 mg of the fresh leaf was taken after being thoroughly washed with distilled water and blotted between folds of tissue paper to remove the moisture. The leaf was ground well and a paste was prepared with a pestle and motor. Distilled water (10 mL) was mixed to the paste and mixed thoroughly to get a fine mixture of extract. The mixture was subsequently centrifuged at x1000 g for 3 minutes to eliminate leaf debris and obtain a clear water extract.

Determination of total phenolic compounds (TPC)

Folin-Ciocalteu method was employed to determine the TPC of leaf extract (Singleton and Rossi, 1965). A 10% 0.5 mL of water mixed Folin-ciocalteu reagent was mixed with 50 µL of water extraction and vortex for about 5 seconds. After 3 minutes, 0.4 mL of 7.5% Na_2CO_3 was added and vortexed again for about 5 seconds and kept for 30 minutes. A blank was prepared similarly but using 50 µL distilled water instead of plant extract. Following a 30-minute interval, the UV-VIS spectrophotometer (SHIMADZU-UV 1800, Japan) was used to measure the absorbance value at 765 nm. Tannic acid, 0.1 g L^{-1}, was used as a standard. The total phenolic content was calculated using equation 1 and was expressed as tannic acid equivalent (mg per g of leaf).

$$TPC = \frac{Absorbance\ of\ extract\ x\ Concentration\ of\ standard}{Absorbance\ of\ standard} \quad (1)$$

DPPH radical scavenging assay

The DPPH activity of *G. sylvestre* extract was measured by the method described by Blois (1958). The 0.135 mM DPPH was prepared in methanol. Different concentration series were prepared by adding different volumes of leaf extract with water and DPPH solution as shown in Table 1.

Table 1: The volume of leaf extract, water and DPPH used for radical scavenging assay

Samples	Extract (µL)	H_2O (µL)	DPPH (µL)
A	250	500	300
B	200	550	300
C	150	600	300
D	100	650	300
E	50	700	300
F	0	750	300

Where A, B, C, D and E were the leaf extract samples and F was the standard solution.

After thorough vortexing, the mixture was incubated in the dark for 30 minutes. The absorbance was taken at 517 nm using sample F as the standard and equation 2 used to calculate scavenging activity.

$$DPPH\ Radical\ Scavenging\ Activity = \frac{(Abs\ control - Abs\ sample)\ x\ 100}{Abs\ control} \quad (2)$$

Where Abs control and Abs samples are absorbance of sample and control, respectively.

Estimation of total antioxidant activity by ferric reducing antioxidant power (FRAP) assay

The total antioxidant power measurement of the *G. sylvestre* leaf extracts was taken by a standard FRAP method (Benzie and Strain, 1999). To prepare a fresh working FRAP reagent, 10 mM of 2, 4, 6-tripyridyl triazine (TPTZ) and 20 mM ferric chloride and 0.25 M acetate buffer (pH 3.6) were mixed in equal portion. Twenty µL of test samples (30 mg mL^{-1}) were mixed to 1 mL of freshly prepared working FRAP reagent in separate test tubes and vortexed well. The 1000 µM $FeSO_4$ solution was the standard in this study. The absorbance was measured after 4 minutes at 593 nm. The total antioxidant activity was calculated using equation 3.

$$Total\ antioxidant\ activity = \frac{Abs\ sample\ x\ Concentration\ of\ standard\ FRAP}{Abs\ standard} \quad (3)$$

Where absorbances of standard and sample were denoted by Abs standard and Abs sample, respectively.

Determination of reducing power

Ferric reducing power of G*. sylvestre* extract was determined as described by Oyaizu (1986) with slight modification. Using *G. sylvestre* extracts, different concentration solutions were prepared with total volume of 400 µL. A mixture of 1 mL of 0.3 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3[Fe(CN)_6]$ was prepared and incubated at 50°C for 20 minutes. Subsequently, a 10 mL trichloroacetic (10%) was mixed with it. Two mL of distilled water and 0.4 mL $FeCl_3.6H_2O$ (1%) were added to this mixture. The absorbance was measured at a wavelength of 700 nm after a 30-minute interval. The standard solution used in this study was tannic acid (0.1 g L^{-1}). The concentration at 50% absorbance was considered as the reducing power of *G. sylvestre* extract. Triplicate samples were measured and the graph was plotted with an average of three observations.

Estimation of monomeric anthocyanin content

The pH differential method was employed to measure monomeric anthocyanin content (Lee *et al.,* 2004). For this purpose, 0.025 M KCl buffer (pH 1.0) and 0.4

M sodium acetate buffer (pH 4.5) were prepared. After 20 minutes, absorbances of corresponding extracts were measured at wave length of 510 and 700 nm. Monomeric anthocyanin content was calculated using equation 4.

$$AC = \frac{A\ x\ MW\ x\ DF\ x\ 1000}{\varepsilon\ x\ 1} \quad (4)$$

Where; AC is Monomeric anthocyanin contents (cyanidin-3-glucoside equivalents, mg L^{-1}), MW is 449.2 g mol^{-1} for cyanidin-3-glucoside, ε equal to 26,900 molar extinction co-efficient, in L mol^{-1} cm^{-1}, for cyd-3-glu, 1 = pathlength in cm, 1000 = conversion factor from g to mg, MW is referred to molecular weight, and DF is referred to dilution factor, and ε is stands for molar absorptivity. The absorbance difference was estimated using equation 5.

$$A = (A535 - A700)_{pH1.0} - (A535 - A700)_{pH4.5} \quad (5)$$

Where A is the absorbance difference at 700 nm and 535 nm for pH 1.0 and pH 4.5.

Statistical Analysis

Results were expressed as mean ± standard deviation. Linear regression analysis was performed to estimate the IC 50 and EC 50 values using Minitab 16 software.

Results and discussion

Total phenolic content

Phenols and phenolic compounds derived from plants are widely used as antioxidants since it is proven that they have antioxidant properties (Rice-Evans *et al.,* 1995). Their scavenging ability is associated with their hydroxyl group (Kessler *et al.,* 2003). The Folin-Ciocalteu method was employed to quantify total polyphenols, which are presented as tannic acid equivalents (mg tannic acid g^{-1}). The TPC of the G*. sylvestre* leaf extract was found to be 4.55±0.34 mg tannic acid equivalent per gramme of FLW. The TPC value for a methanolic leaf extract was reported as 2.2 µg catechol equivalent per mg of FLW (Ahirwal *et al.,* 2013). In another study, the TPC value for G*. sylvestre* leaf extract was reported as 1.31 mg gallic acid equivalent per 100g of dry weight (Surveswaran

et al., 2007). Compared to these values, the present study shows a higher TPC value despite the water being used to get leaf extract than alcohol. This variation of TPC in plant parts could be attributed to environmental factors, location variations, the maturity level of the leaf, rainfall and fertility of the soil (Rajurkar and Hande, 2011).

DPPH radical scavenging ability

This method is very common for studying the free radical quenching ability of a substance (Abhiram *et al.*, 2023). After accepting an electron, stable free radical of DPPH becomes diamagnetic molecule. During the reaction between antioxidant molecules and radicals from DPPH, the hydrogen radical scavenging by the antioxidant causes a decrease in absorbance value and it was measured at 517 nm. This generates a colour change from purple to yellow. The relationship for inhibition percentage of different concentrations of G*. sylvestre* extract was strongly correlated (R^2 = 0.99) (Fig. 1).

The IC50 value was calculated with the following equation derived from the regression analysis: Y = 7.5733X + 31.812 where "X" is the leaf extract concentration and "Y" is the percentage of inhibition. The IC50 value for DPPH radical scavenging ability was 2.4 mg mL^{-1}. The reported value of the DPPH scavenging activity of *G. sylvestre* was 150.6 ± 3.6 µg mL^{-1}(Sahu *et al*., 1996). The present study showed around 16 times lower concentration to inhibit 50% of free radicals compared to the reported value. This shows that a significant amount of the antioxidant was extracted from the leaf using water extract.

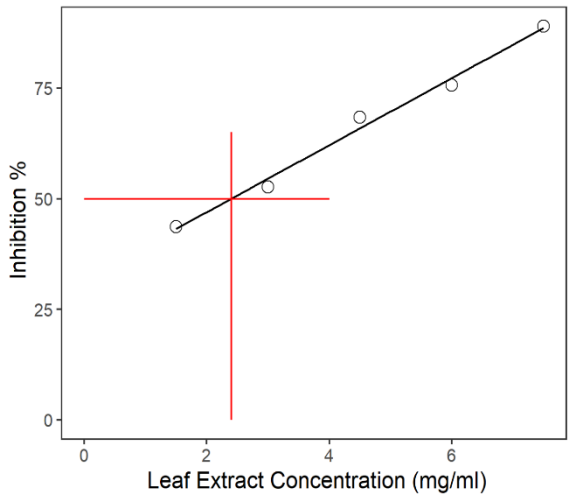

Fig. 1: The inhibition percentage of free radicals by water extract of G. *sylvestre*for DPPH radical scavenging activity. The leaf extract concentration of *G. sylvestre*to inhibit the 50% of DPPH is marked using red colour lines.

Total antioxidant activity

The FRAP assay determined the total antioxidant activity of the leaf extract. FRAP reagent, used in this technique, is responsible for the formation of a blue-coloured Fe II – tripyridyltriazine compound from colourless oxidized Fe III form of the action of electron-donating antioxidants. The antioxidant activities were expressed equivalent to $FeSO_4$ (1000 µM) standard. The total antioxidant power of the *G. sylvestre* leaf extract was 21.1±1.7 µmol g^{-1} of FLW. The total antioxidant activity of *G. sylvestre* extract was reported as 1.69 µmol/ g of DW of the sample and the correlation between the TPC and total antioxidant activity was 0.8941 (Surveswaran *et al.,* 2007). Other studies have reported that *G. sylvestre* extract showed a total antioxidant activity of 9.13±0.04 µg gallic acid equivalent per g FLW (Arunachalam et *al*., 2015) and 17.54 mg per g expressed as ascorbic acid (Rachh *et a*l., 2009).

Reducing power

The antioxidant activity of a plant extract can be evaluated based on its ferric-reducing power, which serves as a valuable indicator (Oyaizu, 1986). In the present study, reducing components in the antioxidant of a leaf extract caused

the reduction of Fe^{3+} (ferricyanide complex) to Fe^{2+} (ferrous). The colour change from yellow to Perl's Prussian blue depends on the reducing power of the leaf extract, which can be measured at 700 nm (Alasalvar *et al*., 2006). Phenolic compounds could be ascribed to the reducing power of the plant extracts (Abhiram *et al.*, 2023). The reducing power was linearly correlated with leaf extract concentration. The aqueous leaf extract showed a concentration-dependent relationship with reducing power (Fig. 2) and it was a strong linear one (R^2 = 0.99). The estimated EC50 value of the aqueous leaf extract was 29.26±0.45 mg ml^{-1}. The tannic acid with reducing power showed a strong correlation (R^2 = 0.99). A similar linear relationship between reducing power and leaf extract of *G. Sylvestre* was reported by Rachh *et al.* (2009). Phenolic compounds are inevitable in quenching oxygen free radical species (Osawa and Osawa, 1994). Reductones breaks larger free radical chain and quench their activity (Gordon, 1990).

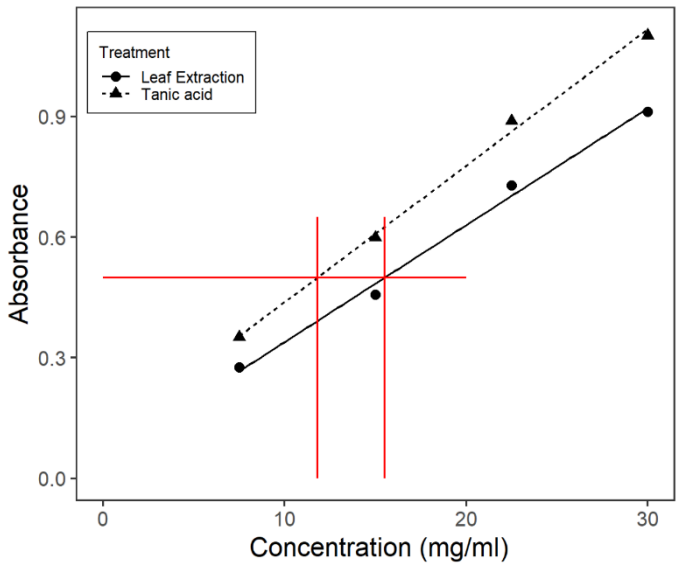

Fig. 2: The reducing power of different concentration of *G. sylvestre*leaf extract and tannic acid. The reducing power of leaf extract is indicated by ▲ and tannic acid is indicated by ■ the symbol. The concentration of leaf extract and tannic acid to inhibit the 0.5 absorbances is marked using red colour lines.

Total monomeric anthocyanin content

The anthocyanin will undergo reversible structural change with pH. At pH 1.0, it exhibits a coloured oxonium form, while at pH 4.5, it takes on a colourless hemiketal form. Based on that anthocyanin has less or no absorbance in pH 4.5 buffer. However, the degraded anthocyanin has an absorbance value of pH 1.0. Hence, the absorbance difference is proportional to the anthocyanin concentration. It was found that the anthocyanin content of G*. sylvestre* leaf extract was 167.84±1.45 mg per 100 g of fresh weight. Total anthocyanin content varies with environmental stress such as ultraviolet light radiation, blue colour radiation, drought, wounding, pathogenic effect and nutrient deficiency (Akula and Ravishankar, 2011). The presence of flavonoids, which includes anthocyanin, in *G. sylvestre* was reported in several studies (Ahirwal *et al.*, 2013; Arunachalam *et al.,* 2015). However, its quantification in *G. sylvestre* was not reported elsewhere. It could be due to their low concentration compared to other phytochemicals.

Correlation of antioxidants (TPC and anthocyanin) with FRAP, DPPH and reducing power assays

The correlation of TPC with FRAP, DPPH and reducing power are shown in Fig. 3 (A, B and C, respectively). All three antioxidant assays showed a positive correlation with total phenolic content. The correlation of coefficient values (R^2) for FRAP, DPPH and reducing power with total phenolic content and anthocyanin content are displayed in Table 2. All correlations were significant other than anthocyanin content and reducing power ($p<0.05$). A strong relationship was observed between TPC and FRAP values. The correlation of phenolic compound and anthocyanin with the different antioxidants showed a common pattern, but with a different coefficient of determination (R^2) values. The possible reasons are: differential responses for antioxidant assays are observed for different phenolic compounds (Kähkönen *et al.,* 1999) and their function is chemical structure dependent (Satué-Gracia *et al.,* 1997).

Both TPC and anthocyanin showed a strong correlation with total antioxidant activity and a weak correlation with reducing power. This insists that total antioxidant activity is a better way to analyze antioxidant activity. The results

of a study of antioxidant properties of 137 Indian medicinal plants also support this inference (Surveswaran *et al.,* 2007). A study based on the fruits' antioxidant activity also suggested that the total antioxidant activity (FRAP) was rapid and simple and high relationship was observed with fruit extract (Thaipong *et al.,* 2006). However, more than one method for assessing antioxidant is merited as it provides the comprehensive view compared to a single method (Číž *et al*., 2010).

Table 2: Correlation of determination (R^2) values for TPC and Anthocyanin with antioxidant assays. The probability of R^2 values are given in parenthesis.

	TPC (mg/g of fresh leaf weight)	**Anthocyanin** (mg/100 g fresh leaf)
Total Antioxidant Activity (FRAP) (µmol/g of fresh leaf weight)	0.9176 (p = 0.0026)	0.9515 (p = 0.0008)
DPPH radical scavenging activity (mg/mL extract)	0.8551 (p = 0.008)	0.9061 (p = 0.003)
Reducing power (mg/mL)	0.7893 (p = 0.01)	0.6242 (p = 0.06)

Fig. 3 (D, E and F) describes the correlation of anthocyanin content with total antioxidant activity (FRAP), DPPH and reducing power, respectively. All the antioxidant assays showed a positive linear relationship with anthocyanin content. There were strong relationships for anthocyanin content with total antioxidant activity and DPPH. Nevertheless, the correlation between anthocyanin and reducing power was weak.

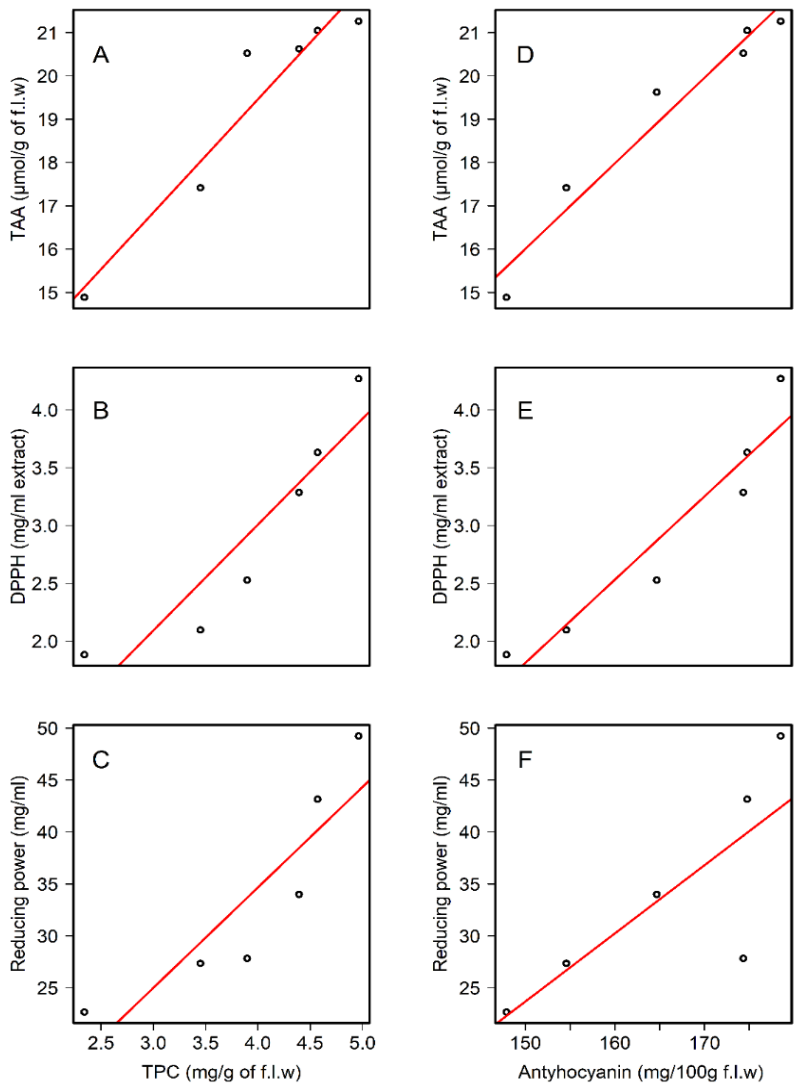

Fig. 3: Correlation between TPC vs. (A) Total antioxidant activity, (B) DPPH radical scavenging and (C) Reducing power and Correlation between Anthocyanin vs. (D) Total antioxidant activity - TAA, (E) DPPH radical scavenging and (F) Reducing power. The abbreviation f.l.w stands for fresh leaf weight.

The correlation between antioxidants present in the leaf extract and antioxidant assays provides confidence to show that these antioxidants take part in antioxidant activity. Few findings were reported on the correlation between total polyphenol and antioxidant assays which purvey the total polyphenol content increases antioxidant function and the relationship was linear (Gorinstein *et al.*, 2009, Číž *et al.*, 2010). This evidenced that polyphenols in *G. sylvestre* leaf significantly contribute to the antioxidant activity. Similarly, a strong relationship between monomeric anthocyanin content and antioxidant assay was reported (Prior *et al.*, 1998). The results of correlation analysis showed that reducing power of plant extracts is well correlated with the phenolic content and it is well reported (Ahirwal *et al.,* 2013, Awika *et al.,* 2003).

Conclusions

This study investigated the antioxidant property of water extract of *G. sylvestre* leaves. The TPC, DPPH radical scavenging, Total Antioxidant Activity by Ferric Reducing Antioxidant Power (FRAP), Ferric reducing power and Total anthocyanin content were measured to describe the antioxidant property. The TPC and anthocyanin content of G*. sylvestre* leaf were high which could be the reason for the antioxidant activity of leaf extract. This evidenced that the water extract of *G. sylvestre* has potent antioxidant properties.

Conflict of interest

No conflict of interests is there to disclose by the authors.

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