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Comparative analysis of antioxidant potential in leaf, stem, and root of *Paederia foetida* L.

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Abstract: *Paederia foetida* L. is widely used for the treatment of myriad ailments. Thus, searching for plant parts having greater antioxidant potential would make it easy to get suitable materials for herbal drugs. The present effort was made to explore the antioxidant potentials in the plant parts of *P. foetida* grown under natural conditions by means of physiological and biochemical analyses. The young leaves showed the highest reservoir of non-enzymatic antioxidants such as chlorophylls (0.96 mg g^{-1}), carotenoids (0.43 mg g^{-1}), anthocyanins (53.99 µg g^{-1}), phenolics (728.24 µg g^{-1}), flavonoids (4178.05 µg g^{-1}), and proline (1.46 µmol g^{-1}) as compared to others. Total antioxidant activity was found to be the highest in young leaves (84.82 %) followed by young stems (80.24 %) and matured leaves (79.78 %). Analysis of enzymatic antioxidants resulted in the superior activity of ascorbate peroxidase (13.58 µmol min^{-1} mg^{-1}) and glutathione S-transferase (3409 nmol min^{-1} mg^{-1}) in young leaves whereas the highest rate of catalase (409.85 µmol min^{-1} mg^{-1}) and peroxidase (3.5 nmol min^{-1} mg^{-1}) activity were found in matured leaves. However, comparatively higher content of reactive oxygen species; hydrogen peroxide, and lipid peroxidation product; malondialdehyde in matured leaves than that of young leaves suggests that young leaf is a suitable source for herbal medicine.

Key words: medicinal plant; antioxidants; free radicals; reactive oxygen species; oxidative stress

Primerjalna analiza antioksidacijskega potenciala listov, stebla in korenin vrste *Paederia foetida* L.

Izvleček: Vrsta *Paederia foetida* L. (smrdljiva trta) se na široko uporablja za blaženje številnih bolezni. Iskanje delov rastline z večjim antioksidacijskim potencialom bi olajšalo pripravo primernih zdravilnih pripravkov. Namen raziskave je bil preučiti antioksidacijski potencial različnih delov rastline, ki je rastla v naravnih razmerah s fiziološkimi in z biokemičnimi analizami. Mladi listi so imeli v primerjavi z drugimi organi največ neencimskih antioksidantov kot so klorofili (0,96 mg g^{-1}), karotenoidi (0,43 mg g^{-1}), antocianini (53,99 µg g^{-1}), fenoli (728,24 µg g^{-1}), flavonoidi (4178,05 µg g^{-1}) in prolin (1,46 µmol g^{-1}). Celokupna antioksidacijska aktivnost je bila največja pri mladih listih (84,82 %), ki so jim sledila mlada stebla (80,24%) in odrasli listi (79,78 %). Analiza encimskih antioksidantov je pokazala največjo aktivnost askorbat peroksidaze (13,58 µmol min^{-1} mg^{-1}) in glutation S-transferaze (3409 nmol min^{-1} mg^{-1}) v mladih listih medtem, ko sta bili aktivnosti katalaze (409,85 µmol min^{-1} mg^{-1}) in peroksidaze (3,5 nmol min^{-1} mg^{-1}) največji v odraslih listih. Primerjalno večje vsebnosti reaktivnih zvrsti kisika, vodikovega peroksida in peroksidacijskih produktov maščob kot je malondialdehid v odraslih listih nakazujejo, da so mladi listi primernejši vir pripravkov pri zdravljenu s to zdravilno rastlino.

Ključne besede: zdravilne rastline; antioksidanti; prosti radikali; reaktivne zvrsti kisika; oksidacijski stres

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1 INTRODUCTION

Medicinal plants possess therapeutic properties and have beneficial pharmacological effects on humans and animals. The medicinal values of these plants are linked with their phytochemical constituents that cause definite pharmacological action in the human body (Khairullah et al., 2021). The phytochemicals such as flavonoids, phenolic acids, isoflavones, carotenoids, phytosterols, saponins, etc. have great antioxidant potential and are of great interest due to their beneficial effects on human health (Thakur et al., 2020). Reactive oxygen species (ROS) are free radicals with one or more unpaired electrons in their outer shell which occur naturally in plants and animals during different metabolic processes (Hasanuzzaman et al., 2020; Adetuyi et al., 2022). Biotic stresses or different ailments and environmental stressors like UV, ionizing radiations, pollutants, heavy metals, and xenobiotics (i.e., antiblastic drugs) enhance the accumulation of ROS in the living cell which have harmful effects on important cellular constituents like proteins, lipids, and nucleic acids (Gómez et al., 2021). Several investigations reported that oxidative stress led by ROS is responsible for the progression of several diseases including cancer, diabetes, metabolic disorders, cardiovascular diseases, arthritis, and stroke, which causing to ultimate cell death in humans (Pizzino et al., 2017; Mahmoud et al., 2021). Human beings set several strategies to counter face the effects of oxidative stress by means of enhanced activities of enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), etc.) and several non-enzymatic antioxidants such as carotenoids, flavonoids, ascorbic acid, α-tocopherol, etc. (Shah and Gupta, 2020). Since endogenous antioxidant defenses are inadequate to mitigate entire damage, antioxidant-rich diets are essential for maintaining good health (Guerra-Araiza et al., 2013). As antioxidants scavenge free radicals from the cells and reduce the damage caused by oxidation, a diet rich in antioxidants might reduce the risk of the above-mentioned diseases and improve overall health conditions. The most familiar exogenous antioxidants are vitamin C, vitamin E, and polyphenols including carotenoids, flavonoids, and phenols (Blázovics, 2022). Although synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tert-butyl hydroquinone are widely used, long-term intake of those resulted in several negative impacts on human health (Engin et al., 2011). So, there has been an increased demand for the therapeutic potentials of plants as natural antioxidant source in reducing oxidative injury. However, indiscriminate use of commonly used commercial antimicrobial drugs against infectious diseases has led to an alarming risk in advancing resistance to multiple drugs, thus necessitating the need for searching the alternatives source of synthetic drugs from medicinal plants (Bungau et al., 2021; Hassan et al., 2021). Antimicrobial drugs of plant origin have enormous therapeutic potential and show a higher level of efficacy in the treatment of infectious diseases rather than synthetic drugs having enormous side effects. However, scientists found that foods containing phytochemicals with antioxidant potential have strong protective effects against the risks of cancer and cardiovascular diseases (Okoye, 2021). Therefore, developing a potential herbal source for beneficial phytochemicals and antioxidants is of great interest to the scientific community for sustainable health security.

Paederia foetida (Skunk vine or Gondhabadali) plant belongs to the family Rubiaceae, is a widely distributed medicinal plant in Asia and especially in south-east Asia (Okamoto et al., 2008). It has a broad spectrum of uses in the treatment of ailments like hepatic disorders, rheumatoid arthritis, constipation, diabetes, coughs, asthma, itches, wounds, stomachache, diarrhoea, dysentery, pain, typhoid, pneumonia, toothache, cancer, etc. (Soni et al., 2013). The presence of essential plant metabolites having anti-ulcer, anti-diarrhoeal, antihyperglycemic, antioxidant, antitussive, and anthelmintic activity in *P. foetida* (Soni et al., 2013) suggests the plant is a potential reservoir of herbal drugs. Although some recent investigations suggest the antioxidative, antidiabetic, and antimicrobial efficacy of *P. foetida* (Karmakar et al., 2020; Satapathy and Pattnaik, 2020; Ghosh et al., 2021a), comparative analysis of antioxidants emphasizing entire plant parts is still to be clarified. However, analysis of plant parts having the best enzymatic antioxidant potential is still to be reported in this valuable medicinal plant. Additionally, the natural occurrence of ROS in the plant parts is yet to be determined. Therefore, the present investigation was set to identify the suitable plant part of *P. foetida* with the best enzymatic and non-enzymatic potentials through physiological and biochemical assays. The findings suggest that the young leaf of *P. foetida* is the best source of antioxidants and utilization of which should mitigate the effect of excess ROS produced by ailments and environmental stressors. Thus, the findings might help develop suitable herbal drugs and maintain sustainable health security.

2 MATERIALS AND METHODS

2.1 COLLECTION AND PREPARATION OF PLANT MATERIALS

The stem cuttings of *P. foetida* were grown in the research field of Bangabandhu Sheikh Mujibur Rah-

man Agricultural University and those were allowed to grow naturally with proper care and management. The primary branching of the healthy and suitable plants was selected for collecting plant samples. The fresh and fully expanded young leaves (YL) of 15 days-aged, matured leaves (ML) of 45 days-aged, young stems (YS) of 15 days-aged, matured stems (MS) of 45 days-aged, and root (R) of 45 days-aged of *P. foetida* were collected from the selected branches. After repeated washing, the collected materials were allowed to dry for 4-5 days in an oven. The dried materials were chopped and crushed into powder and stored in air-tight containers for further analyses.

2.2 CHLOROPHYLLS AND CAROTENOIDS CONTENT DETERMINATION

Chlorophylls content from freshly collected leaf and stem tissues and carotenoids content from leaf, stem and root tissues were determined using the method described by Porra et al. (1989). Briefly, 100 mg of plant tissues were taken in a glass vial and 5 ml of 80 % acetone was added. The vials were made airtight and kept at 4 °C in the dark for 24 hours. After extraction, the separated plant extracts were taken to measure the absorbance through a spectrophotometer at 663, 646, and 470 nm wavelengths respectively. Blank measurement was done using only acetone. The quantification was done according to the formula of Lichtenthaler & Welburn (1983). The chlorophylls and carotenoids content were expressed as milligram per gram of fresh sample (mg g^{-1}).

2.3 ANTHOCYANINS CONTENT DETERMINATION

Anthocyanins content was determined with little modifications as described by Hughes and Smith (2007). Briefly, 1 g shade-dried powder of plant parts was taken in an ice-cold glass vial containing 5 ml methanol. After making the vials airtight, those were kept in dark condition for 24 hours. Then, 2 ml of extracts were centrifuged with 2 ml distilled water and 2 ml chloroform at 5000g at 4 °C for 15 minutes. The absorbance was measured at 530 nm. Quantification was done according to the formula of Murray and Hackett (1991). The anthocyanin content was expressed as microgram of cyanidin-3-glucoside equivalent per gram of dry sample (µg g^{-1}).

2.4 DETERMINATION OF PHENOLICS CONTENT

Phenolics content of the methanolic extracts was determined spectrophotometrically according to the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007). The absorbance of reaction solutions was measured at 765 nm against a blank sample. Quantification was done according to the formula of Abdul-Hafeez et al. (2014). The measurements were compared to a standard curve of gallic acid solutions and expressed as micrograms of gallic acid equivalents per gram dry mass (µg g^{-1}).

2.5 DETERMINATION OF FLAVONOIDS CONTENT

The methanolic extract of plant materials was used for the determination of flavonoids content using the aluminium-chloride colorimetric assay (John et al., 2014). Quercetin at different concentrations was used as the standard solution. The absorbance of the extracts and standard solutions was measured at 510 nm using a UV/Visible spectrophotometer. The results were expressed as micrograms of quercetin equivalents (QE) per gram of dry mass (µg g^{-1}).

2.6 DETERMINATION OF ENZYMATIC ANTIOXIDANT ACTIVITY

Fresh plant tissues of the plant materials (0.5 g) were homogenized in 1 ml extraction buffer containing 1 mM ascorbic acid, 1 M KCl, 0.5 M K-P buffer (pH 7.0), β-mercaptoethanol and glycerol in ice-cold mortar and pestle. The homogenates were centrifuged at 11,500 × g for 15 min, and the supernatant was used as a soluble protein solution for enzyme activity. The protein concentration was determined by the method of Bradford (Bradford, 1976) using BSA as a protein standard. The catalase (CAT) activity was measured according to the method of Hasanuzzaman et al. (2014). The activity of CAT was determined as µmol min^{-1} mg^{-1} protein using the extinction coefficient of 39.4 M^{-1} cm^{-1}. The activity of ascorbate peroxidase (APX) was assessed by following the procedure outlined by Nakano & Asada (1981). The activity of APX was determined as µmol min^{-1} mg^{-1} protein using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. The activity of peroxidase (POD) was measured by following the method of Hemeda and Klein (1990). The activity of

POD was determined as nmol min^{-1} mg^{-1} protein using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. Glutathione S-transferase (GST) activity was measured by following the procedure of Hossain et al. (2010). The activity of GST was determined as nmol min^{-1} mg^{-1} protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

2.7 DETERMINATION OF ANTIOXIDANT ACTIVITY (% DPPH SCAVENGING ACTIVITY) AND PROLINE CONTENT

The plant extracts were supposed to showing DPPH radical scavenging activity by following the method of Abdul-Hafeez et al. (2014). Ascorbic acid was used to make a reference solution. The following equation was used to get the inhibition percentage:

% DPPH radical scavenging activity = $[(A_0 - A_1) / A_0] \times 100$. Where A_0 = absorbance of the control and A_1 = absorbance of the sample. The proline content in different plant parts was measured spectrophotometrically using the acid ninhydrin assay, as described by Bates et al. (1973). The proline content was determined as µmol g^{-1} fresh mass using a standard curve.

2.8 DETERMINATION OF HYDROGEN PEROXIDE (H_2O_2) AND MALONDIALDEHYDE (MDA) CONTENT

The H_2O_2 content was measured following the method of Ghosh et al. (2021b). The leaf samples (0.1 g) were homogenized in 1.5 ml 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500 × g at 4 °C for 15 min. 0.08 ml of supernatant was taken and added 0.2 ml of 100 mM KP buffer (pH 7.5) and 0.8 ml of KI. The tubes were kept in ice for 1 hour and then at room temperature for 20 min to stabilize the reaction. The absorbance was measured at 390 nm. The concentration of H_2O_2 was calculated by using the absorption coefficient of 156 mM^{-1} cm^{-1} and expressed as µmol g^{-1} fresh mass (FM).

MDA content was measured by using thiobarbituric acid (TBA) as the reactive material following the method of Ghosh et al. (2021b). The absorbance was measured at 532 nm and 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 156 mM^{-1} cm^{-1} and expressed as µmol of MDA g^{-1} fresh mass.

2.9 STATISTICAL ANALYSIS

All the experiments were conducted by following CRD (Completely Randomized Design) with four replications. Statistical analysis was performed using Statistix 10 software. The least significant difference (LSD) value at 5 % level of significance and student t-test were used for showing significant differences. The correlation coefficient matrix was visualized using the 'metan' package of R software.

3 RESULTS AND DISCUSSION

3.1 ACCUMULATION OF CHLOROPHYLL PIGMENTS VARIES WITH THE DEVELOPMENTAL STAGES OF PLANT PARTS IN *P. foetida*

Since chlorophylls (chl) as one of the important primary metabolites regulating ROS *in vitro* (Vaňková et al., 2018; Choi et al., 2016), we determined and compared chlorophylls contents in the plant parts of *P. foetida*. This study revealed that the young leaf showed the highest chl content than other parts. *The young leaf, matured leaf, young stem, and matured stem contained 0.62 mg g^{-1}, 0.51 mg g^{-1}, 0.14 mg g^{-1}, and 0.11 mg g^{-1} chl a respectively* (Fig. 1a). In case of chl *b*, the young leaf, matured leaf, young stem, and matured stem contained 0.222 mg g^{-1}, 0.177 mg g^{-1}, 0.067 mg $g^{-1,}$ and 0.059 mg g^{-1} chl *b* respectively where the young leaves had the highest chl *b* followed by the matured leaf, young stem, and matured stem (Fig. 1b). The total chl content in the young leaf, matured leaf, young stem, and matured stem was 0.964 mg g^{-1}, 0.784 mg g^{-1}, 0.240 mg $g^{-1,}$ and 0.198 mg g^{-1} respectively. Likewise, in chl *a* and chl *b*, the young leaves had the highest chl content followed by the matured leaves, young stems, and matured stems (Fig. 1c). We also calculated the ratio of chl *a* and chl *b* (chl *a*/*b*) and found the ratio of 2.826:1, 3.064:1, 2.291:1, and 1.977:1 in young leaves, matured leaves, young stems, and matured stems respectively (Fig. 1d).

Along with being photosynthetic pigments, *chlorophylls* are naturally strong *antioxidants* acting as free radical scavengers (Nazarudin et al., 2022; Pérez-Gálvez et al., 2020). The previous investigation found that chlorophyll extract of *Sauropus androgynous* (L.) is vital to protect against the consequences of oxidative stress in rats (Suparmi et al., 2016), suggesting that the plant parts potentiated with high chlorophyll content should be essential for managing ROS in animals' ailments. The previous investigation reported that Chl content increased from tender leaf, peaked in photosynthetically matured leaf, and then further declined after getting its maximum (Czech et al., 2009). In our case, the highest chlorophyll content in fully expanded young leaf (YL) agreed with that finding (Fig. 1). The total chl content in the leaf of *P.*

foetida was found as 1.2643 ± 0.0396 mg g^{-1} FM in *P. foetida* (Nayak et al., 2015) which was consistent to the results of the present study (Fig. 1). The Chl *a*, chl *b,* and total chl contents of the leaves of *P. foetida* were also measured by Islam et al. (2018) and the results showed little variation than the present study (Fig. 1) which may be due to varied growing, developing, and climatic conditions. The fresh leaves of twenty-one medicinal plants comprising trees, shrubs, and herbs, were investigated for quantification of chlorophyll content which resulted in a wide variation in chl *a*, chl *b,* total chl content, and chl *a/b* ratio (Ghosh et al., 2018). Along with those findings, our observation on the variation of chlorophyll content in different plant parts of *P. foetida* (Fig. 1) suggests that chlorophyll content varies with the species, age, and developmental stages of the plant. The highest chlorophyll content in the young leaf rather than older matured leaf, and the young and matured stem indicates the potential parts of *P. foetida* having greater antioxidant potential.

3.2 VARIED ACCUMULATION OF CAROTENOIDS IN THE PLANT PARTS OF *P. foetida*

Since carotenoids are established as the crucial metabolites for having strong antioxidants (Pérez-Gálvez et al., 2020), we determined those in the plant parts. The carotenoid content was found the highest (0.43 mg g^{-1}) in the young leaves which significantly differed from the other parts. Matured leaves had the second-highest carotenoid content which was 0.28 mg g^{-1}. Carotenoid content of the young stem and matured stem were 0.16 mg g^{-1} and 0.17 mg g^{-1} respectively which were nearly similar and there was no significant difference between them. Roots had the lowest carotenoid content which was 0.058 mg g^{-1} (Fig. 2). The findings of the previous investigations showed higher carotenoids content in the leaf of *P. foetida* (Ghosh et al., 2021a; Islam et al., 2018) which makes an agreement to the present findings where higher carotenes content resulted in the leaves (Fig. 2). A little variation of

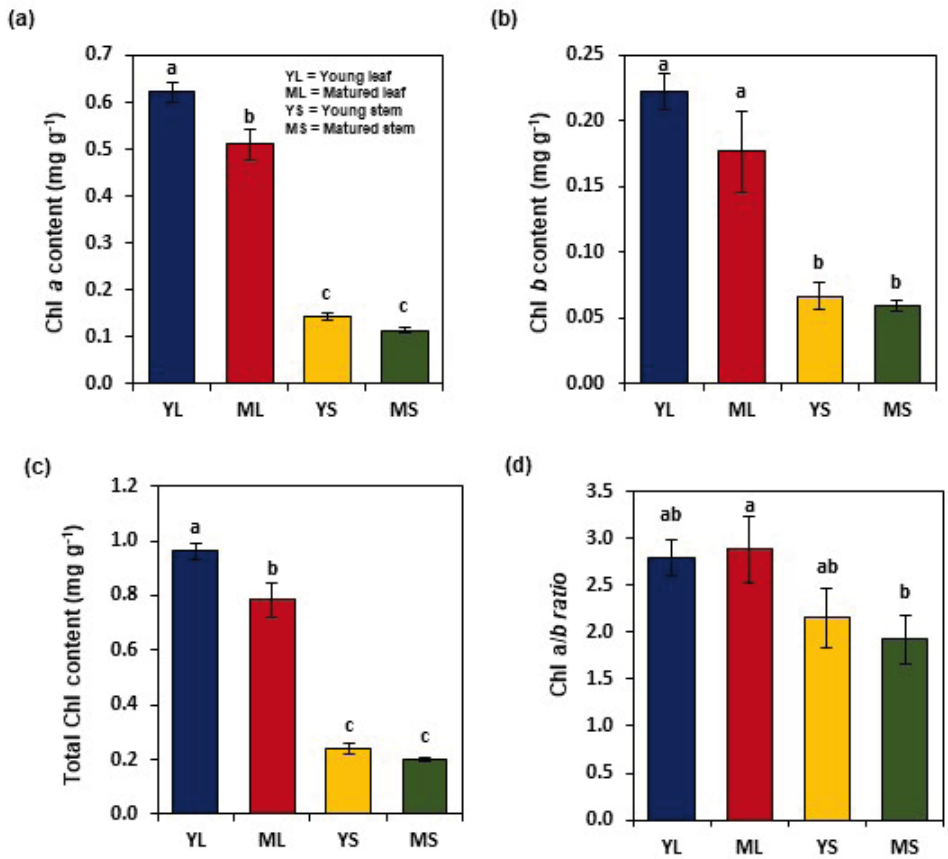

Figure 1: (a) Chl *a* content, (b) Chl *b* content, (c) Total chl content, and (d) Chl *a/b* ratio in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, and MS denote young leaf, matured leaf, young stem, and matured stem respectively

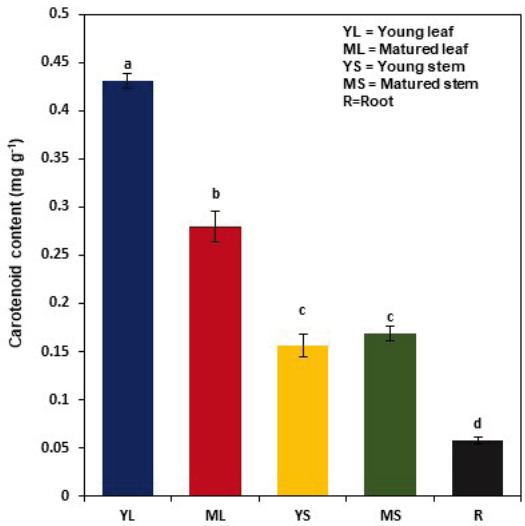

Figure 2: Carotenoid content in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, MS, and R denote young leaf, matured leaf, young stem, matured stem, and root respectively

carotenoids content was reported by Nayak et al. (2015) which may be due to the difference in growing conditions, cultivar, and assay techniques. Carotenoid content was reported in the methanolic extracts of different plant parts of *Hypericum foliosum* Aiton including young leaves, old leaves, stem bark, stems, root, seed capsules, and flowers (Rainha et al., 2011). The study claimed that total carotenoids were found higher in stem and stem bark followed by leaf, root, and seed. In contrast, our study regarding various levels of carotenoids in different plant parts of *P. foetida* (Fig. 2) suggests that the plant is a good source of carotenoids where young leaves have greater efficacy than other plant parts.

3.3 THE PLANT PARTS OF *P. foetida* SHOW VARIATION IN THE ACCUMULATION OF POLYPHENOLS

As polyphenols possess very good antioxidative and pharmaceutical properties (Khan et al., 2021), we determined total phenolics, anthocyanins and flavonoids contents and compared those in the plant parts of *P. foetida*. The young leaves contained the highest amount of phenolics (728.243 μg g^{-1}) followed by the matured leaves (667.945 μg g^{-1}), young stems (651.748 μg g^{-1}), matured stems (589.455 μg g^{-1}), and roots (442.178 μg g^{-1}) (Fig. 3a). Phenolics has been reported to be very effective against cardiovascular diseases by means of having anti-inflammatory, antioxidants, and antiplatelet effects (Khan et al., 2021). A lot of investigations reported the presence of total phenolics in different medicinal plants (Sharma et al., 2022; Pandey and Sharma, 2022; Alfarrayeh et al., 2022; Khan et al., 2022;) and the results of which are consistent with our findings (Fig. 3a). Phenolics content in *P. foetida* was greatly affected by plants parts where leaf accumulated higher phenolics than that of stem and root (Ghosh et al., 2021a). The phenolic contents in this study showed little variation from the findings of others with *P. foetida* (Rosli et al., 2021; Ojha et al., 2018). The variation is due to the age and growing conditions of the plants. As the young leaf of *P. foetida* is very sensitive and more heavily exposed to stressful conditions than other plant parts, it accumulates higher phenolics for better protection against environmental stresses. Along with the findings of the above-mentioned studies, the result of the present study indicates that *P. foetida* is a good source of phenolic contents.

Along with phenolic contents, the anthocyanins content also varied in the plant parts of *P. foetida* in this study (Fig. 3b). The young leaves contained the highest amount of anthocyanins which was 53.99 μg g^{-1} followed by the young stems, matured leaves, matured stems, and roots which contained 39.02 μg g^{-1}, 26.48 μg g^{-1}, 22.09 μg g^{-1}, and 11.67 μg g^{-1} anthocyanins respectively. Anthocyanins are a family of natural pigments considered to be responsible for the color and taste of many fruits and vegetables (Zhang and Jing, 2022; Sunil and Shetty, 2022; Bocker and Silva, 2022). Anthocyanins found in different fruits were reported to have strong antioxidant and anti-inflammatory properties which could inhibit lipid peroxidation (Reis et al., 2016). Previous investigations in other medicinal plants resulted in the presence of this metabolite at various ranges (Sharma et al., 2022; Puzerytė et al., 2022; Joshi et al., 2017). As anthocyanins are a pigment molecule and related to sunlight, roots were found to have the lowest amount of anthocyanin (Fig. 3b). Very recent efforts on *P. foetida* suggested that as compared to roots both leaves and stems are good sources of anthocyanins (Ghosh et al., 2021a). Along with these, the highest level of anthocyanins in the young leaves (Fig. 3b) suggesting that the leaves of *P. foetida* are a good reservoir of anthocyanins.

The young leaves also showed significantly higher flavonoids content than other plant parts (Fig. 3c). There was no significant difference between the flavonoids content of matured stems and roots. The flavonoids contents in the young leaves, matured leaves, young stems, matured stems, and roots were 4178.053 μg g^{-1}, 3871.95 μg g^{-1}, 2662.075 μg g^{-1}, 1372.71 μg g^{-1}, and 1258.22 μg g^{-1} respectively (Fig. 3c). Due to photosynthesis in leaves,

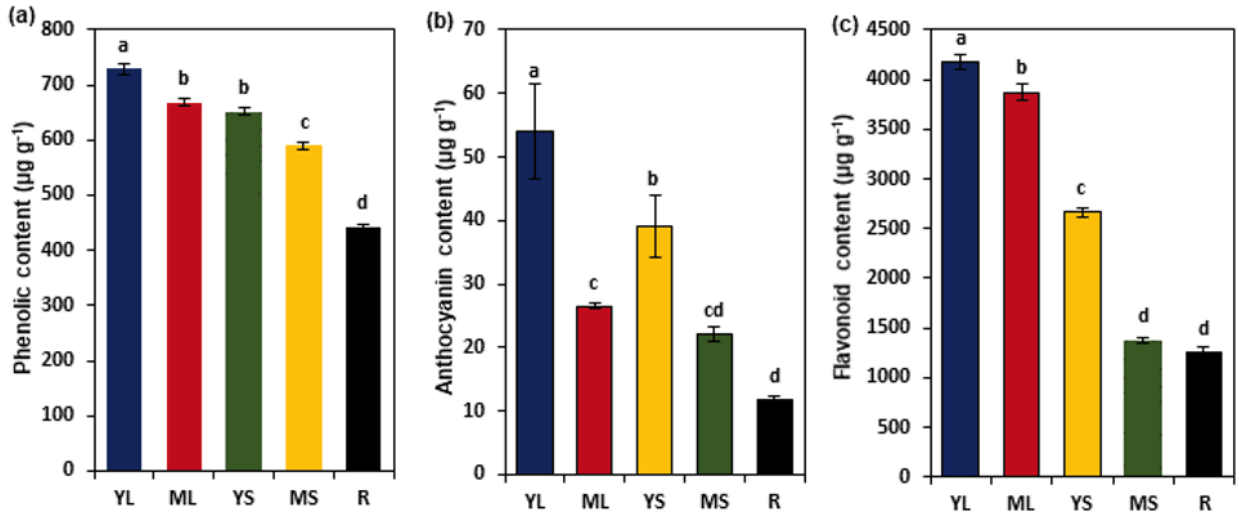

Figure 3: (a) Phenolics (b) anthocyanins, and (c) flavonoids contents in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, MS, and R denote young leaf, matured leaf, young stem, matured stem, and root respectively

flavonoids biosynthetic pathway precursors are more abundant in leaves (Andersen and Markham, 2005). So, flavonoids content was higher in the leaves of *P. foetida* than in other plant parts. Flavonoids content was also reported in *P. foetida* in several studies (Ghosh et al., 2021a, Rosli et al., 2021; Karmakar et al., 2020). Along with those, our findings regarding higher flavonoids content in both young and matured leaves (Fig. 3c) support that the leaves irrespective of whether young or matured are the best source of flavonoids in *P. foetida*.

3.4 ACTIVITY OF ENZYMATIC ANTIOXIDANTS IN THE PLANT PARTS OF *P. foetida*

Enzymatic antioxidants play a crucial role in mitigating the negative impacts of free radicals in cellular and metabolic processes (Hasanuzzaman et al., 2020), thus we determined the activity of CAT, GST, APX, and POD in different plant parts of *P. foetida*. Since matured stems and roots showed lower performances in non-antioxidant activity (Figs. 2 & 3), we focused on the young leaves, matured leaves, and young stems for the determination of enzymatic antioxidant activity.

It was found that the matured leaves showed the highest CAT activity followed by the young leaves and young stems (Fig. 4a). The young leaves, matured leaves, and young stems had the catalase activity of 189.420 µmol min^{-1} mg^{-1}, 409.852 µmol min^{-1} mg^{-1}, and 96.910 µmol min^{-1} mg^{-1} protein respectively. In contrast to CAT activity, APX activity was found to be the highest in the young leaves (Fig. 4b). The young leaves, matured leaves, and young stems showed APX activity as 13.58 µmol min^{-1} mg^{-1}, 8.51 µmol min^{-1} mg^{-1}, and 3.905 µmol min^{-1} mg^{-1} protein respectively (Fig. 4b). The activity of POD was found to be the highest in matured leaves followed by the young stems, and young leaves (Fig. 4c). Young leaves, matured leaves, and young stems showed POD activity as 1.3 nmol min^{-1} mg^{-1}, 3.5 nmol min^{-1} mg^{-1}, and 2.5 nmol min^{-1} mg^{-1} protein respectively. On the other hand, the young leaves showed the highest Glutathione S-transferase (GST) activity than the other parts (Fig. 4d). The young leaves, matured leaves, and young stems had the GST activity of 3039.697 nmol min^{-1} mg^{-1}, 774.568 nmol min^{-1} mg^{-1}, and 167 nmol min^{-1} mg^{-1} protein respectively.

In the enzymatic defense system, catalase (CAT) is very ubiquitous to all living organisms which catalyzes the decomposition of hydrogen peroxide into water and oxygen (Vitolo, 2021). The enzyme is very crucial for defending cells against oxidative damage. The enhanced activity of SOD and CAT during oxidative stress in rat by the exogenous application of aqueous root bark, stem bark and leaves extracts of *Vitex doniana (Adetoro et al., 2013) suggesting the potentiality of plant extracts in mitigating oxidative stress in animals*. Nayak et al. (2015) found no induction of CAT activity in the leaves of *P. foetida*, the result of which was incompatible with our findings where CAT activity was greatly induced in all the plant parts (Fig. 4a). This may be due to the difference in growing conditions, cultivar, and assay techniques. Our study was supported by several investigations where CAT activity was sufficiently reported in medicinal plants (Güneş et

al., 2019; Kumar et al., 2012). Along with that, the higher CAT accumulation in the matured leaves of *P. foetida* (Fig. 4a) rather than that of the younger leaves suggesting the activity of CAT varies with the developmental phases of plants. However, the presence of a higher level of CAT in both young and matured leaves suggests that the leaves of *P. foetida* might be a potential source of exogenous catalase. The key member of the ascorbate reduced glutathione (ASA-GSH) cycle, APX was reported to protect chloroplasts and other cell constituents from damage caused by hydrogen peroxide and hydroxyl radicals (Asada, 1992). Though APX activity was reported in medicinal plants (Güneş et al., 2019; Kumar et al., 2012), no previous study regarding the determination of APX activity was made in *P. foetida*. However, a higher level of APX accumulation in *P. foetida* supporting the potential source of this enzymatic antioxidant where the young leaves showed better potential than others (Fig. 4b). Alongside, POD which represents a family of isoenzymes is actively involved in oxidizing ROS (Khan et al., 2014). Though the incidence of POD activity was reported in the medicinal plant species by previous investigation (Güneş et al., 2019), the natural occurrence of POD in *P. foetida* was not reported so far. The variation of POD activity in different plant parts of *P. foetida* implies that the plant is a very good source of exogenous POD, where matured leaves showed better potential than other plant parts. Along with the above-mentioned antioxidants, GST is another key antioxidant enzyme that can quench reactive molecules with the addition of glutathione (GSH) and protect the cell from oxidative damage (Kumar and Trivedi, 2018). Although enzymatic antioxidant GST has been reported in the stress acclimation of land plants (Horváth et al., 2015; Labrou et al., 2015), the natural occurrence of GST was unexplored in medicinal plants. However, GST accumulation in the plant parts

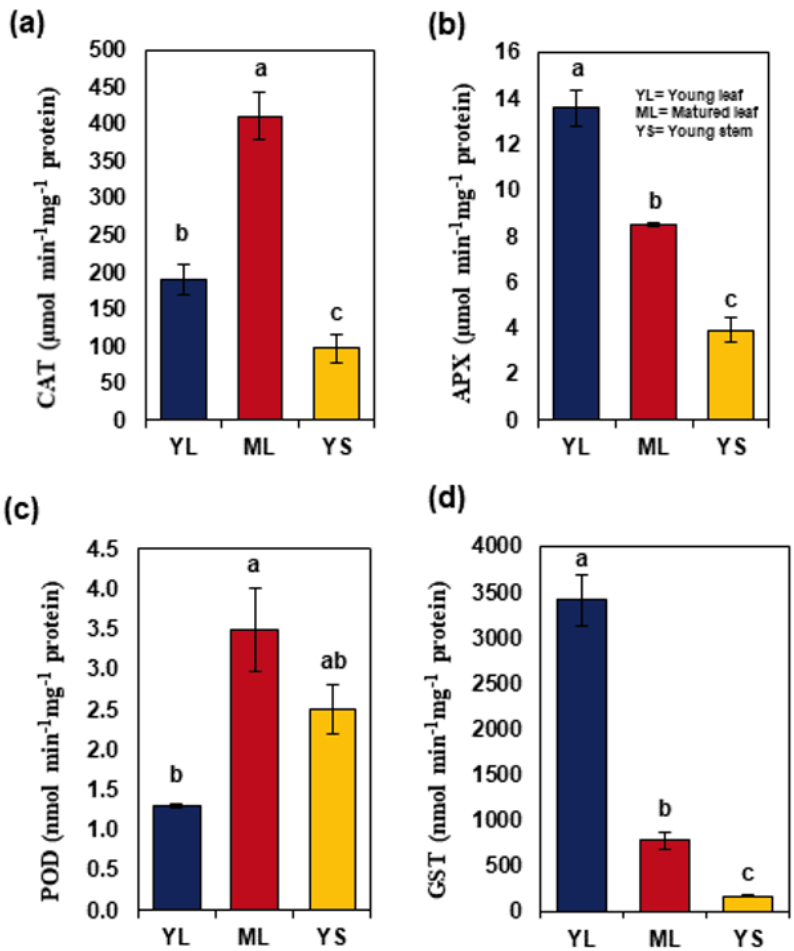

Figure 4: (a) Catalase, (b) ascorbate peroxidase, (c) peroxidase, and (d) glutathione S-transferase in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, and YS denote young leaf, matured leaf, and young stem respectively

of *P. foetida* suggesting the potent natural source of GST where young leaves showed better potential than others.

3.5 TOTAL ANTIOXIDANT ACTIVITY (% DPPH SCAVENGING ACTIVITY) IN THE PLANT PARTS OF *P. foetida*

The antioxidant activity in terms of % DPPH scavenging activity in the plant parts of *P. foetida* was recorded as 84.82 %, 79.78 %, 80.24 %, 48.18 %, and 23.37 % respectively (Fig. 5). So, we could see that the younger portion of the plant; young leaves and stems were very rich in antioxidant activity. According to Sahoo and Bhatnagar (2015), a significant antioxidant activity of 84-85 % was reported in *P. foetida*, the results of which agreed with the results of the present study where both leaves and stems showed about 80 % DPPH scavenging activity (Fig. 5). Similar observation was recorded by another effort in *P. foetida* (Upadhya, 2013). In contrast to fresh leaves, the shade-dried leaves of *P. foetida* exhibited a dose-dependent DPPH free radical scavenging manner, where about 60 % inhibition was recorded by 500 mg ml^{-1} *P. foetida* extract (Uddin et al., 2014). Rutnakornpituk and Boonlue (2013) recorded 74.72 % DPPH scavenging activity in ethyl acetate crude extract of *P. foetida* and the results of which were consistent with the present study. Along with those, in our observation, although no significant differences were found among the young leaves, matured leaves and young stems, the young leaves showed the highest level of total antioxidant activity by means of % DPPH scavenging ability (Fig. 5) and data of which were consistent to the increased levels of non-enzymatic and enzymatic antioxidants in young leaves (Figs. 1, 2, 3, 4).

3.6 PROLINE CONTENT IN THE PLANT PARTS OF *P. foetida*

Since osmolyte proline acts as an antioxidant and has been found to directly react with ROS (Kaul et al., 2008, Sharma and Dietz, 2009), we determined proline accumulation in the plant parts of *P. foetida*. The young leaves contained the proline content of 1.465 µmol g^{-1} followed by the young stems (1.105 µmol g^{-1}) and matured leaves (0.974 µmol g^{-1}) (Fig. 6). Osmolyte proline is frequently employed as a non-enzymatic antioxidant to combat the negative effects of various ROS and attributed as an efficient scavenger of hydroxyl radicals and singlet oxygen (Naliwajski and Skłodowska, 2021). However, proline could act as an antioxidant and be involved in the protection of oxidative damage in a wide array of organisms including fungi, plants, and animals (Krishnan

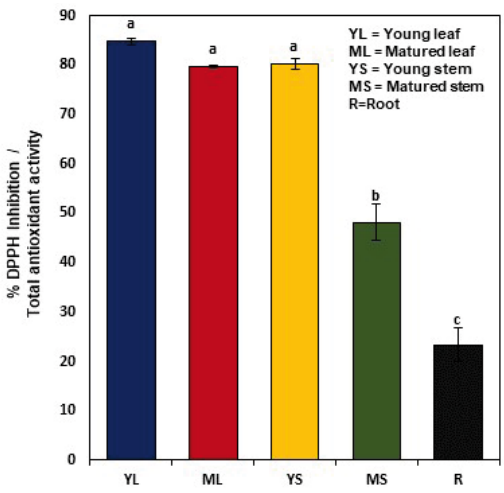

Figure 5: Total antioxidant activity or % DPPH scavenging activity in different parts of *P. foetida*. Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, YS, MS, and R denote young leaf, matured leaf, young stem, matured stem, and root respectively

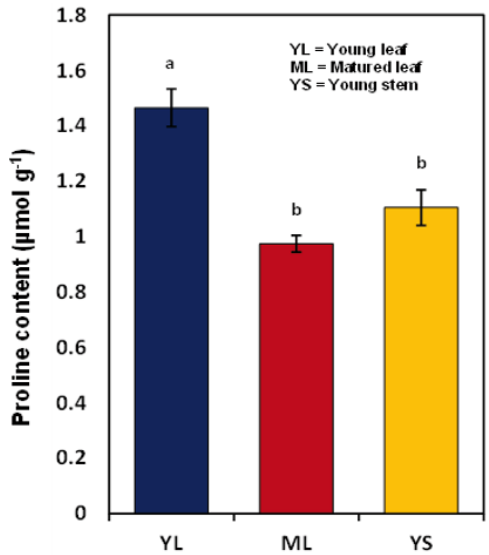

Figure 6: Proline content in different parts of *P. foetida.* Values (mean ± SE) of each treatment were attained from four replications. Error bars indicate standard error. Different alphabetical letters on the bars show significant differences ($p < 0.05$) among the treatments following a least significant difference test. YL, ML, and YS denote young leaf, matured leaf, and young stem respectively

et al., 2008, Chen et al., 2005). Additionally, proline was shown to be reported in the protection of human skin cells from photo-oxidative stress suggesting that proline is essential for human ailments (Wondrak et al., 2005). Although proline accumulation in non-stressed conditions was not emphasized more in medicinal plants, a very recent study showed a higher amount of proline accumulation in vulnerable and threatened medicinal plants *Blepharis sindica* T. Anders (Lal et al., 2021). In our study, the highest proline accumulation was found in young leaves rather than matured leaves and stems under non-stressed conditions. The variation of accumulation is due to the various role of proline in the plant's ontogenic process (Kishor et al., 2015). As proline is crucial for living organisms including plants and animals, the plant parts having greater proline accumulation would be good reservoir for herbal drugs.

3.7 H_2O_2 AND MDA CONTENT

To compare the occurrence of ROS in young and matured leaves, we measured H_2O_2 and lipid peroxidation product MDA in those plant parts. The matured leaves had higher H_2O_2 and MDA contents (28.75 µmol g^{-1} FM and 28.60 µmol g^{-1} FM respectively) than in young leaves (27.66 µmol g^{-1} FM and 23.51 µmol g^{-1} FM respectively) (Fig. 7). Though there was no significant difference in H_2O_2 content of young and matured leaves, they show significant difference in case of MDA content at 5 % level of significance. ROS is naturally produced in the plant's body and at lower concentrations acts as signalling molecules in response to growth, development and stress responses, whereas appears as detrimental at higher concentrations (Huang et al., 2019). H_2O_2 is one of the most important members of ROS and enhanced accumulation of which causes lipid peroxidation and membrane injury in plants (Sachdev et al., 2021; Hemantaranjan et al., 2014). MDA is an indicator of lipid peroxidation and oxidative stress which causes membrane leakage (Nahar et al., 2022; Tsikas, 2017). Along with plants, oxidative stress is linked with the occurrence of many human diseases including cancer, brain misfunctioning, diabetes, heart disease, etc. (Law et al., 2017). Therefore, plant parts having a lower occurrence of ROS might be suitable for health concerning issues. In our study, the higher accumulation of H_2O_2 in matured leaves than in young leaves was consistent with the elevated level of MDA in matured leaves (Fig. 7).

3.8 CORRELATION ANALYSIS

The relationship between antioxidative parameters (chlorophyll *a*, chlorophyll *b*, total chlorophylls, chlorophyll *a/b* ratio, carotenoids, anthocyanins, phenolics, flavonoids, DPPH scavenging activity, proline, CAT, GST, APX, POD) was determined through the values of the correlation coefficient where positive values were indicated as red and negative values as blue. The relationship ranged from -1 to 1, whereby -1 means a perfect negative and 1 means a perfect positive linear relationship between variables and 0 indicated no relationship between studied variables (Fig. 8). The results indicated a signifi-

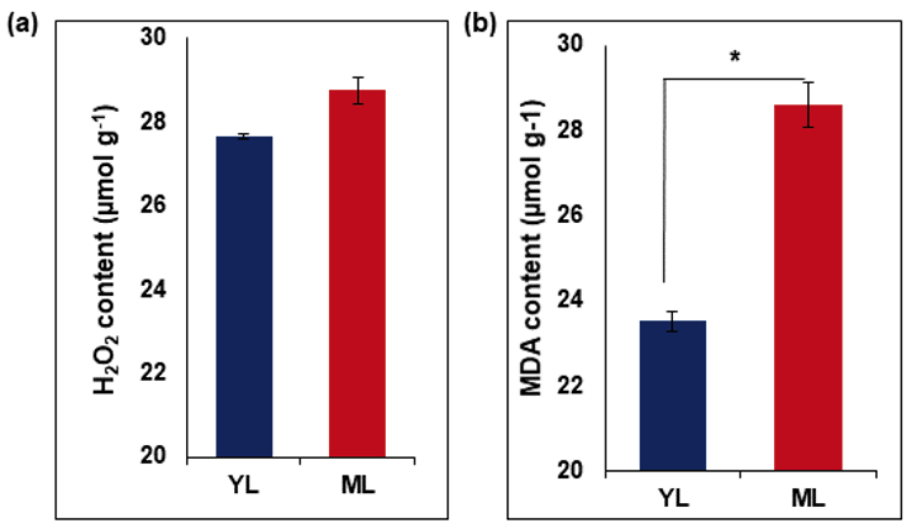

Figure 7: (a) H_2O_2 content and (b) MDA content in young and matured leaves of *P. foetida.* Values (mean ± SE) of each treatment were attained from four replications. Aster mark indicates significant difference between the treatments ($p < 0.05$). YL and ML denote young leaves and matured leaves respectively

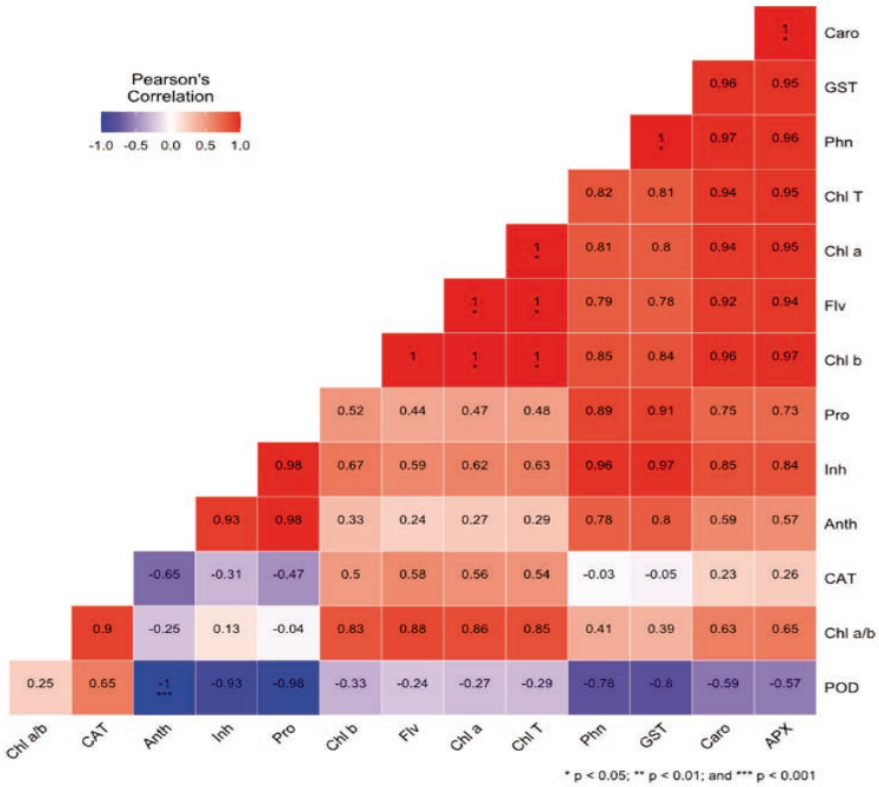

Figure 8. Correlation analysis for showing relationship between antioxidative parameters. The parameters included Chl *a* (chlorophyll *a*), Chl *b* (chlorophyll *b*), Chl T (total chlorophylls), Chl *a*/*b* (chlorophyll *a*/*b* ratio), Caro (carotenoids), Anth (anthocyanins), Phn (phenolics), Flv (flavonoids), Inh (% inhibition of DPPH; Total antioxidant activity), Pro (proline), CAT (catalase), GST (glutathione S-transferase), APX (ascorbate peroxidase), and POD (Peroxidase). The positive values are in red, and the negative values are in blue. It ranges from -1 to 1, whereby -1 means a perfect negative and 1 means a perfect positive linear relationship between variables and 0 indicates no relationship between studied variables

cant positive correlation between various antioxidative parameters. Among all phytochemical parameters, chl *a* chl *b*, total chl, and flavonoids were positively and highly correlated (r = 1) at 5 % level of significance. Carotenoids content was positively and highly correlated with APX (r = 1) and negatively correlated with POD (r = -0.59). GST and phenolics were also highly correlated (r = 1) at 5 % significance level. CAT was almost positively correlated with APX and POD and negatively with GST. Anthocyanins content was negatively correlated with POD and CAT. Carotenoids, APX, flavonoids, chlorophylls, phenolics and total antioxidants were positively correlated with each other. Although some paraments showed a negative correlation with each other, most of them maintained a positive correlation with total antioxidant activity in terms of % DPPH scavenging activity. Our findings are consistent with the findings of others where Chl *a* and Chl *b* contents of stem amaranth made a positive correlation with total antioxidant activity (Sarker et al., 2020). Likewise, phenolic compounds in plants made a positive relationship with the antioxidant activity of the tissue (Doğan et al., 2014; Güne et al., 2019). In our observation, positive correlation among most of the parameters (Fig. 8) suggesting that both enzymatic and non-enzymatic antioxidants in the plant parts of *P. foetida* contribute synergistically for boosting up the total antioxidant activity in the plant.

4 CONCLUSIONS

According to the present study, young leaves showed the best potential for non-enzymatic antioxidants like chlorophylls, carotenoids, anthocyanins, phenolics, flavonoids, and proline. Among enzymatic antioxidants, GST and APX activity was found to be the highest in young leaves whereas CAT and POD activity were superior in matured leaves. Although there were no significant differences, the total antioxidant activity in terms of % DPPH scavenging activity was found the highest in young leaves followed by young stems and matured leaves. Based on overall observation, it can be concluded

that the medicinal plant *P. foetida* is a very good source of both enzymatic and non-enzymatic antioxidants. The young leaf of the plant might be a suitable option for the preparation of the natural herbal drug. However, further *in vitro* and *in vivo* studies with animal models are required to see the efficacy of the plant parts as potential human drugs.

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