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Total phenolic content and antioxidant activities in methanol extracts of medicinal herbs from Indo-Gangetic plains of India

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ABSTRACT

The present study investigates total phenolics, flavonoids, and *in vitro* antioxidant activities in methanol extracts of roots, stems, leaves, and fruits of medicinal herbs (*Phyllanthus fraternus* G.L. Webster; Bhui-amla and *Solanum nigrum* L.; Makoi or black nightshade) of Indo-Gangetic plains of India. The results showed that biochemical attributes of methanol extracts of both the tested plants varied significantly with sites ($P < 0.05$). Contents of total phenolics and flavonoids and antioxidant properties were found highest in fruits followed by leaf, stem, and least in root of *P. fraternus* and leaves, fruits, stem, and roots of *S. nigrum* plants. The present investigation revealed that total phenolic content in fruits of *P. fraternus* ranged from 26.69 to 61.48 mg GAE/g fw and from 8.89 to 24.69 mg GAE/g fw in leaves of *S. nigrum* plants, and thus, these plant parts can be promoted for pharmaceutical purposes and health benefits. It is also suggested that the individual phenolic compound in the different parts of tested plants should be analyzed to identify their elite population for their mass cultivation, conservation, and sustainable utilization.

1. INTRODUCTION

The scientific community has been encouraged for long time to investigate the medicinal potential of herbs for identification of potential source of natural antioxidants both in the field of herbal medicines and food industries [1]. Mostly herbal medicines are a mixture of a variety of ingredients from plants whose combined effect enhances their effectiveness in the treatment of diseases. Traditional uses of herbal therapy have been recognized to be safe and are frequently utilized for curing long-term diseases. Many medicines with plant origins are used in current pharmacotherapy [2]. Since the prehistoric past, people have recognized that medicinal plants can be used to prevent disease, and this knowledge has been passed from generation to generation within human groups. Tribal people were familiar with the therapeutic powers of natural herbs [3]. Compared to synthetic drugs, herbal medicine has a larger potential for healing diseases because of their synergistic effects [4]. Thousands of herbal and natural compounds are being studied globally to check their use as antioxidants.

Various antioxidants found in plants used in traditional medicine, such as polyphenols, carotenoids, tocopherols, glutathione, ascorbic acid, and antioxidant enzymes, support the body's defense mechanism against harmful oxidative damage [5]. Allopathic medicine is increasingly utilizing these polyphenols, which are secondary metabolites, to help maintain human health by acting as antispasmodic and tumor-prevention agents and antioxidants. [6]. The majority of flavonoids have anti-inflammatory, antioxidant, and anticancer properties [7]. Phenolic substances have the ability to quench oxygen-derived free radicals by giving them a molecule of hydrogen or a single electron, which gives them antioxidant activity. The ability of medicinal plants to act as antioxidants may be related to phenolic substances, such as flavonoids, phenol anthocyanins, and polyphenols. The development of new and potent natural antioxidants benefits the pharmaceutical industry [8]. Modern technology has the potential to extract antioxidants from both edible and non-edible parts of fruits and vegetables, which is favorable for diet inclusion and contributes to global good health [9]. *Phyllanthus fraternus* G.L. Webster (Euphorbiaceae) is a medicinal herb of great significance as possesses antiinflammatory, hypoglycemic, antihepatotoxic, and antidiarrheal activities and is rich in terpenoids, flavonoids, phenolic acids, stilbenes, anthocyanins, coumarins, and lignins, as well as other polyphenolic chemicals [10]. *Solanum nigrum* L. (Solanaceae) is a common herb and traditionally used as a hepatoprotective agent in India [11]. *S. nigrum* is one of the richest sources of anthocyanins [12]. In Mexican medicine, the fruit of *S. nigrum* is utilized as a nervous tonic.

Phytochemical analysis of both *P. fraternus* and *S. nigrum* plants is necessary to unlock their full utilization potential in medicine, drug development, and standardizing herbal products [13,14]. Such analysis contributes to our understanding of plants health benefits, safety, and

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environmental applications, making it a valuable and multidisciplinary field of investigations. These plants have been chemically analyzed, and compounds such as solasodine, solasonine, and solanidine have been identified [15]. Furthermore, the antioxidant, antiulcer, and anticancer qualities of this plant's fruit are being utilized historically [16]. Soil physicochemical characteristics exert a complex and multifaceted influence on the accumulation of different phytochemicals such as phenolics and flavonoids in plants. Understanding these intricate relationships is crucial for optimizing plant production and enhancing the nutritional and medicinal properties of plant-derived products. A useful secondary metabolite that supports plant health, human welfare, and sustainable agricultural practices may be produced by adapting such farming practices to accommodate certain soil conditions. Although numerous reports are available regarding the total phenolics and total flavonoids content, as well as antioxidant efficacy of stem, leaves and fruits of both the *P. fraternus* and *S. nigrum* plants. However, root of these tested plants is unexplored further effect of soil physicochemical properties on the biochemical attributes is studied. Therefore, primary objective of the present study was to assess total phenolic content and antioxidant activities in methanol extracts of medicinal herbs, *P. fraternus and S. nigrum.* which naturally grow in the Varanasi region of Indo-Gangetic plains, Northern India with the intention of examining the spatial distribution of biochemical attributes. The relationship of biochemical attributes of methanol extracts with physico-chemical properties of growing media, i.e., soil was further investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

Analytical-grade chemicals, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tri-2-pyridyl-1,3,5-triazine (TPTZ), Folin-Ciocalteu phenol reagent, gallic acid, quercetin, ascorbic acid, sodium carbonate, methanol, and aluminum chloride, were procured from Merck Millipore, India, for use in the study.

2.2. Sampling and Processing

Whole parts of *P. fraternus* and *S. nigrum* plants along with soil were collected randomly in triplicates during July to September 2022 from five different sites, namely BHU agriculture farm, Babatpur, Mohansarai Railway Station, Chaukhandi, and Mirzamurad (S1, S2, S3, S4 and S5, respectively) located in different areas of varanasi, India [Figure 1 and Table 1]. The plant samples have been gathered and brought to the laboratory. They got properly cleaned with tap water to remove any dirt or contaminants. Subsequently, the samples were manually separated into different parts, including leaves, stems, fruits, and roots. These separated parts were then air-dried until a constant weight was achieved, ensuring that all moisture was removed. Once dried, the plant parts were crushed into a fine powder using stainless steel grinder mixer. To achieve a consistent and fine texture, the final product has been passed using a sieve with a mesh size of 2 mm. Finally, the sieved powder had been preserved at the ambient temperature, and it was ready for biochemical analysis.

2.3. Soil Analysis

0.25 g air-dried samples of soil were utilized for heavy metal analysis. These soil samples were digested using a mixture of 10 mL of 70% pure HNO_3 and 65% $HClO_4$ in a 9:4 ratio at a temperature of 80°C. The digestion process continued until a transparent solution was obtained, using the method outlined by Jackson [17]. After digestion, the resulting Whatman No. 42 filter paper was used to filter the solution. The resulting filtrate was subsequently diluted to a final amount of 25 ml with distilled water. An atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800, USA) was used to evaluate heavy metal contents in soil samples. The amounts of all heavy metals in the samples were utilized to determine the geometric mean using a defined formula to estimate the heavy metal pollution index (MPI).

$$MPI = (C_1 \times C_2 \times C_3 \times ....\times C_n)^{1/n}$$

Here, C_n stands for the concentration of n heavy metals in the sample.

2.4. Preparation of Methanol Extract and Analysis

Fresh weight of each plant part of *P. fraternus* and *S. nigrum* plants was used for the preparation of methanol (80% v/v) extract. To make it, 2 g of each sample was crushed into 20 ml of methanol and they were kept for 48 h at 4°C. Subsequently, the mixture was subjected to centrifugation at 10,000 g for 10 min at room temperature. Following the centrifugation process, the liquid portion containing the intended substances, known as supernatants, was meticulously gathered and preserved in a refrigerator at 4°C for subsequent analysis.

2.4.1. Determination of total phenolic contents

Total phenolic content in the leaf, stem, root, and fruit methanol extracts of the tested plants was quantified using the Folin-Ciocalteu phenol reagent method after slight modification [18]. Briefly, 1 ml of plant extract was blended with 1 ml of the 1N phenol reagent Folin-Ciocalteu and 2 ml of 7% (w/v) sodium carbonate, and the final volume of reaction mixture was maintained to 10 ml using double distilled water. Then, the reaction mixture was heated for 30 min at 80°C in a water bath till blue color appeared strongly. The optical density of the blue-colored mixture at 760 nm was measured using a spectrophotometer (UV-Vis Spectrophotometer, Model no 2203, Systronics, India). A standard curve was prepared using different concentrations of gallic acid (0–100 µg/ml) and data were expressed as mg GAE (gallic acid equivalents)/g fw.

2.4.2. Determination of total flavonoid contents

Total flavonoid content in leaf, stem, root, and fruit methanol extract from tested plants was quantified using an aluminum chloride reagent as per the method described by Ordonez *et al.* [19]. One milliliter of plant extract was mixed thoroughly with 1 ml of 2% ethanolic $AlCl_3$ (w/v). The reaction mixture was allowed to stand at 25°C for 1 h. The optical density of golden yellow colored was measured 420 nm using a spectrophotometer (UV-Vis Spectrophotometer, Model no 2203, Systronics, India). Quercetin (0–100 µg/ml) was used to prepare the standard curve. The total flavonoid content was expressed as mg quercetin equivalent (QE)/g fw in plant extracts.

2.4.3. DPPH radical assay

A method was used to evaluate the DPPH radical scavenging activity in the methanol extract of leaf, stem, root, and fruit of both *P. fraternus* and *S. nigrum* plants using method described by Liyana-Pathirana and Shahidi [20]. One milliliter of plant extract was mixed with 5 ml of DPPH (0.135 mM), freshly prepared in 80% (v/v) methanol and the reaction mixtures were incubated for 30 min at room temperature in the dark. Optical density of the reaction mixture was read at 517 nm using a spectrophotometer (UV-Vis Spectrophotometer, Model no 2203, Systronics, India). One milliliter of methanol mixed with 5 ml of DPPH was taken as blank. The DPPH inhibition potential of different extracts expressed in percentage (%) was measured using the following equation.

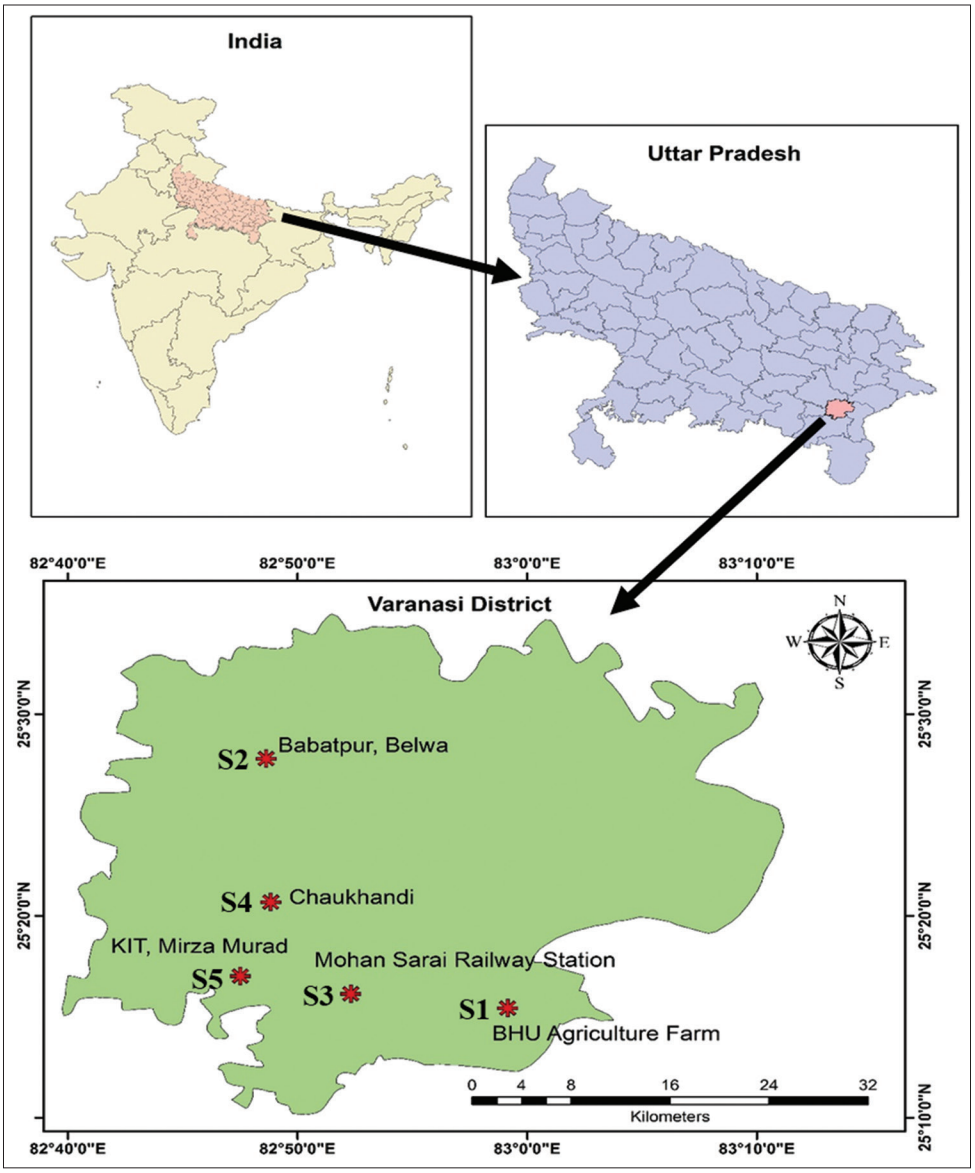

Figure 1: Map showing the location of different study sites, located in the Varanasi region of Indo-Gangetic plains of India.

Table 1: Selected characteristics of study sites located in the Varanasi region of Indo-Gangetic plains of India.

Study sites	Site ID	Traffic load	Population density	Land use
BHU	S1	Low, Link roads	Sparse	Agriculture
Babatpur	S2	Moderate, National highway	Sparse	Residential
Mohan Sarai	S3	Heavy, National highway	Dense	Transport
Chaukhandi	S4	Heavy, Link roads	Dense	Transport
Mirzamurad	S5	Heavy, National highway	Dense	Transport

$$\text{DPPH Inhibition \%} = (A_b - As/A_b) \times 100$$

Where,
A_b = Absorbance of the blank,
A_s = Absorbance of the sample.

2.4.4. ABTS radical assay

ABTS radical scavenging in methanol extract of leaf, stem, root, and fruit of both *P. fraternus* and *S. nigrum* plants using the method described by Re *et al.* [21]. Equal volumes of 7 mM ABTS solution and 2.45 mM potassium persulfate solution were mixed for the production of ABTS radicals. The reaction mixture was kept at room temperature in the dark and then diluted ABTS cation using 80% (v/v) methanol to achieve 0.700 ± 0.02 absorption at 734 nm. Five milliliter of diluted ABTS solution was mixed with 1 ml of plant extract. The absorbance of the reaction mixture was read at 734 nm after 5 min of incubation using a spectrophotometer (UV-Vis Spectrophotometer,

Model no 2203, Systronics, India). The ABTS scavenging activity in plant extracts expressed in percentage (%) was computed using the following equation.

$$\text{ABTS scavenging activity (\%)} = (A_b - A_s/A_b) \times 100$$

Where,
A_b= Absorbance of the blank,
A_s= Absorbance of the sample.

2.4.5. FRAP assay

The FRAP activity in methanol extract of the leaf, stem, root, and fruit parts of *P. fraternus* and *S. nigrum* plants was measured using the method of Benzie and Strain *et al*. [22]. A reaction mixture containing 10 mM TPTZ in 40 mM HCl, 1 ml of 20 mM ferric chloride, and 10 mL of 300 mM acetate buffer (3.1 g of sodium acetate and 16 ml of glacial acetic acid per liter) were prepared and pre-heated at 35°C. The plant extract (150 µl) was added to 3 ml of the above reaction mixture and kept at room temperature for 10 min. The optical density of the reaction mixture was measured at 593 nm A using a spectrophotometer (UV-vis spectrophotometer, model no 2203, Systronics, India). The ferrous sulfate solution was used for the preparation of standard curve and the results were expressed as µg Fe II/g fw.

2.5. Statistical Analysis

A three-factor analysis of variance (ANOVA) was executed to explore the separate and collective impacts of different independent factors, namely Plant Species (PS), Plant Parts (PP), and Sites (S) on the biochemical attributes data. The acquired data underwent statistical analysis, and the outcomes were exhibited as average value accompanied by the standard error of three replications ($n = 3$). For the purpose of identifying statistically significant differences among the means, Duncan's multiple range test was utilized with a significance threshold set at $P < 0.05$. Moreover, regression analyses were conducted to evaluate the correlation between soil heavy metal concentrations and the biochemical characteristics of distinct plant components. All the statistical evaluations, encompassing ANOVA, Duncan's test, and regression analyses, were executed utilizing SPSS version 16 software.

3. RESULTS

The two medicinal herbs, *P. fraternus* and *S. nigrum*, which were naturally growing in various locations within the Varanasi region of Uttar Pradesh, India, were found to be abundant in naturally occurring phenolics and flavonoids. Moreover, these plants exhibited strong antioxidant activities. The total phenolic and flavonoid content in the methanol extracts of the tested medicinal herbs, as well as their antioxidant activities, showed significant variations across different sites [Tables 2 and 3] with a significance level of $P < 0.05$. The results suggest that particular locations where the plants are growing the levels of phenolics, flavonoids, and antioxidant activities varied significantly. The finding indicated that the total phenolic content in *P. fraternus* and *S. nigrum* plants grown in Varanasi exhibited a wide range, with values varying from 3.55 mg GAE/g fw in the roots at site S1 to 58.86 mg GAE/g fw in the fruits at site S5 for *P. fraternus*, and from 0.89 mg GAE/g fw in the stems at site S1 to 23.88 mg GAE/g fw in the leaves at site S5 for *S. nigrum* [Table 2].

Among the plant parts, the richest total phenolic content was estimated in the fruits (61.48 GAE/g fw) of *P. fraternus* and in the leaves (24.69 GAE/g fw) of *S. nigrum*, indicating that these parts had the highest concentration of total phenolic compounds in the respective plants [Table 2].

The total flavonoid contents in the tested plants exhibited a range from 0.072 mg QEs/g of fresh weight (fw) in the roots of *P. fraternus* at site S1 to 0.543 mg QE/g fw in the fruits at site S5. Similarly, for *S. nigrum*, the total flavonoid content varied from 0.032 mg QE/g fw in the roots at site S2 to 0.335 mg QE/g fw in the leaves at site S5 [Table 3]. The roots of both *P. fraternus* and *S. nigrum* contained the lowest amounts of total flavonoids, with values of 0.071 mg QE/g fw and 0.02 mg QE/g fw, respectively. On the other hand, the fruits of *P. fraternus* and the leaves of *S. nigrum* had the highest amounts of total flavonoids, with values of 0.561 mg QE/g fw and 0.34 mg QE/g fw, respectively [Table 3]. Furthermore, the total phenolic content in the fruits of *P. fraternus* and the total flavonoid content in the leaves of *S. nigrum* showed a decreasing trend across the different sites, with the highest content observed at site S1 and the lowest at site S5 [Tables 2 and 3]. Additionally, the total flavonoid content in the fruits of *P. fraternus* and the leaves of *S. nigrum* was found to be highest at site S5, followed by S3, S4, S2, and least at site S1 [Table 3].

Radar plots were employed to visually compare the levels of heavy metals – namely Cu, Cd, Cr, Ni, and Zn – across various locations [Figure 2]. The radar diagrams revealed that the sources of heavy metal pollution at each location varied significantly ($p<0.05$). Among the sites, S5 exhibited the highest concentrations of all heavy metals in comparison to the rest.

Regression analysis was carried out between the soil MPI and the biochemical activities of the root, stem, leaf, and fruit of *P. fraternus* and *S. nigrum* [Figure 3]. The relationship between soil MPI and biochemical activities has been assessed with the R^2 value. In terms of total phenolics, the maximum and minimum R^2 values were reported in *S. nigrum* leaf ($R^2 = 0.90$) and root ($R^2 = 0.77$), respectively, rather than *P. fraternus* leaf ($R^2 = 0.86$) and fruit ($R^2 = 0.75$), respectively. In case of total flavonoids, the maximum and minimum R^2 were found in *P. fraternus* leaf ($R^2 = 0.89$) and stem ($R^2 = 0.71$), respectively, rather than *S. nigrum* fruit ($R^2 = 0.86$) and root ($R^2 = 0.80$), respectively. In case of DPPH, ABTS, and FRAP, the maximum and minimum R^2 value was found to be in *P. fraternus* fruit ($R^2 = 0.92$), root ($R^2 = 0.72$), and leaf ($R^2 = 0.91$), fruit ($R^2 = 0.84$), and leaf ($R^2 = 0.90$), stem ($R^2 = 0.86$), respectively, in present study, the correlation between soil MPI and biochemical activities were statistically significant ($P < 0.001$). The

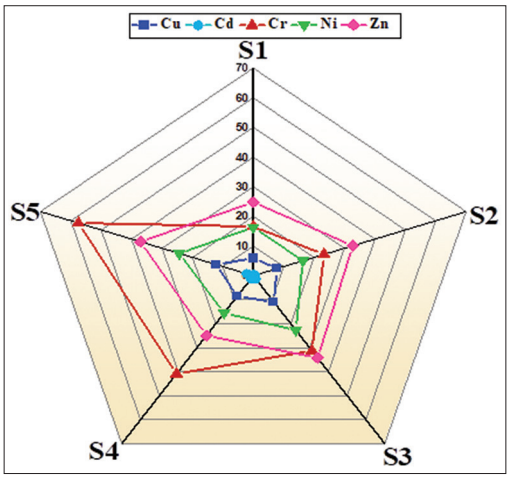

Figure 2: Spider or radar graph showing concentrations of different heavy metals at different sites located in the Varanasi region of Indo-Gangetic plains of India.

Table 2: Total phenolics content in different part of *P. fraternus* and *S. nigrum* plants collected from different study sites located in Varanasi region of Indo-Gangetic plains of India.

Sampling locations	Total phenolics content (mg GAE/g fw)							
	P. fraternus				*S. nigrum*			
	Root	Stem	Leaf	Fruit	Root	Stem	leaf	Fruit
S1	3.55^{d}	4.39^{d}	9.33^{e}	27.11^{c}	0.89^{c}	2.86^{a}	9.43^{d}	2.96^{c}
S2	4.24^{c}	4.66^{d}	12.62^{d}	43.46^{b}	1.04^{c}	1.04^{c}	10.50^{d}	4.36^{b}
S3	6.29^{a}	7.20^{b}	25.04^{b}	57.17^{a}	2.86^{a}	3.31^{a}	18.78^{b}	5.21^{b}
S4	5.64^{b}	5.95^{c}	15.32^{c}	44.18^{b}	1.7^{b}	1.70^{b}	14.21^{c}	4.45^{b}
S5	6.64^{a}	9.26^{a}	42.45^{a}	58.86^{a}	3.31^{a}	1.57^{b}	23.88^{a}	6.82^{a}
Average	5.27	6.30	20.93	46.15	1.96	3.88	15.36	4.76
Min.	3.39	3.22	8.93	26.69	0.84	1.95	8.89	2.55
Max.	7.02	9.76	44.44	61.48	3.74	6.27	24.69	6.91

Values are mean of three replicates. Standard error for each site was below 10% of its mean value. Values in each column followed by different letters are significantly different at $P<0.05$.

Table 3: Total flavonoids content in different part of *P. fraternus* and *S. nigrum* plants collected from different study sites located in the Varanasi region of Indo-Gangetic plains of India.

Sampling locations	Total flavonoids content (mg QE/g fw)							
	P. fraternus				*S. nigrum*			
	Root	Stem	Leaf	Fruit	Root	Stem	leaf	Fruit
S1	0.072^{e}	0.213^{d}	0.325^{c}	0.432^{d}	0.036^{e}	0.059^{e}	0.283^{e}	0.110^{d}
S2	0.091^{d}	0.302^{c}	0.352^{c}	0.481^{c}	0.032^{d}	0.065^{d}	0.305^{d}	0.131^{c}
S3	0.133^{b}	0.341^{ab}	0.461^{a}	0.520^{b}	0.060^{b}	0.105^{b}	0.320^{b}	0.164^{b}
S4	0.124^{c}	0.334^{b}	0.410^{c}	0.492^{c}	0.035^{c}	0.069^{c}	0.313^{c}	0.136^{c}
S5	0.142^{a}	0.352^{a}	0.502^{a}	0.543^{a}	0.064^{a}	0.171^{a}	0.335^{a}	0.201^{a}
Average	0.111	0.310	0.413	0.492	0.04	0.09	0.31	0.15
Min.	0.071	0.213	0.284	0.431	0.02	0.06	0.28	0.11
Max.	0.141	0.373	0.511	0.561	0.07	0.17	0.34	0.21

Values are mean of three replicates. Standard error for each site was below 10% of its mean value. Values in each column followed by different letters are significantly different at $P<0.05$.

Pearson correlation coefficient, which demonstrates the strength of the linear relationship between two variables, is displayed as a heat map in the correlation matrix [Figure 4].

The information gathered through PCA analysis is expanded upon by the correlation matrix heat map. In case of *S. nigrum* plant, a strong positive correlation was detected between root TF with stem DPPH ($R^2 = 0.991$) as well as between root TF with root DPPH ($R^2 = 0.98$). The minimum positive correlation was found between stem TF with stem ABTS ($R^2 = 0.71$). There was found strong negative correlation between soil PH and soil organic matter with other variables of all biochemical attributes of *S. nigrum.* Soil organic matter showed strong negative correlation with stem TF ($R^2 = 0.72$) and weak correlation with root ABTS ($R^2 = 0.97$). Stem TF had strong negative ($R^2 = 0.79$) and stem DPPH showed weak negative ($R^2 = 0.95$) correlations with soil PH. Furthermore, in case of *P. fraternus*, no significant correlation between root TF with stem DPPH and soil pH with fruit DPPH was found. Stem TP showed a strong positive correlation with leaf DPPH ($R^2 = 0.94$) although leaf TP has found poor positive correlation with root TP ($R^2 = 0.18$).

The association between different biochemical attributes and heavy metals content in rhizospheric soil of *P. fraternus* and *S. nigrum* was determined using the principal component analysis. Results of PCA analysis revealed that the first principal axes accounted for 94.1% of the variance and followed by the second principal axes (4.77%) [Figure 5a]. PCA for the heavy metal pollution index is represented by PC 1 and PC2 (89.6% and 9.7%, respectively) [Figure 5b].

The antioxidant capacities of the methanol extracts of the roots, stems, leaves, and fruits of both tested plants were assessed using three different assays: DPPH, ABTS, and FRAP [Figure 6].

The results obtained from these assays are presented as percentages for DPPH and ABTS, and as micrograms of Fe (II) per gram of fresh weight (fw) for FRAP [Figure 6]. DPPH, ABTS and FRAP were highest in fruit methanol extract *P. fraternus* at S5 (72.61%, 88.04 % and 70.43 µg Fe II/g fw, respectively) and leaf extract of *S. nigrum* at S5 (67.71%, 87.21% and 39.50 µg Fe II/g fw, respectively). DPPH, ABTS and FRAP activities in different methanol extracts of *P. fraternus* varied from 23.2% to 72.6 %, 55.1% to 88.1% and 11.8 µg Fe II/g fw to 70.4 µg Fe II/g fw, respectively, and 9.3 % to 67.7%, 52.1 % to 87.2 % and 4.1 µg Fe II/g fw to 39.5 µg Fe II/g fw, respectively in *S. nigrum* [Figure 6].

The present study further revealed that in *P. fraternus,* the maximum total phenolic, total flavonoid, ABTS, and FRAP were found in leaf except DPPH in fruits. However, in case of *S. nigrum* total phenolic, DPPH and FRAP were maximally reported in leaf except total

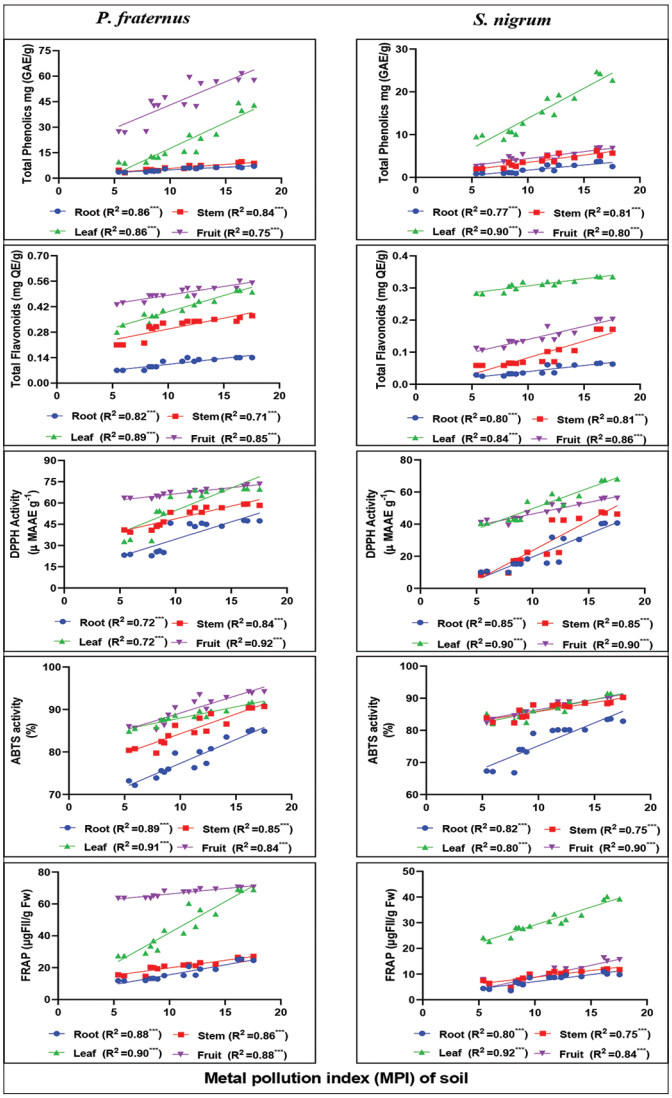

Figure 3: Regression correlation between metal pollution index of soil and biochemical characteristics of methanol extract obtained from different parts of *P. fraternus* and *S. nigrum.* Levels of significance **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and ns.

flavonoid and ABTS in fruits. The present study also estimated that soil heavy metal causes test plant leaves to have higher biochemical activities than fruit, stems, and roots, ascribed to more metabolic activities in leaves.

4. DISCUSSION

The overall phenolic and flavonoid content in the methanolic extracts of root, stem, leaf, and fruit of medicinal plants, i.e., *P. fraternus* L. and *S. nigrum* L. were assessed. The occurrence of flavonoids, phenolics, and antioxidant properties in the *P. fraternus* plant extract was discovered through phytochemical analysis [23]. Plants have therapeutic capabilities because they contain a variety of complicated chemical compounds that are only found in specific areas of the plant and have a strong healing effect on the human body. Bioactive components and antioxidant properties of *P. fraternus* have been the area of research to justify the claims of traditional healers. Research and development in the emerging pharmaceutical field is at the pick. Polyphenolic metabolites found in plants, such as flavonoids and phenolics, have been shown to have a variety of biological effects, including antioxidant activity. According to Peng *et al.* [24], poly-phenolic compounds are responsible for beneficial antioxidant activities in the *S. nigrum* extracts. "The present study showed that both the tested plants were good sources of antioxidant substances. In the present study, total phenolic content varied between 3.55 mg GAE/g fw (Root) to 58.86 mg GAE/g fw (Fruit) in *P. freternus* and 0.89 mg GAE/g fw (Stem) to 23.88 mg GAE/g fw (Leaf) in *S. nigrum*, respectively [Table 2]. The current study reveals that the total phenol content in the leaf extracts of *P. fraternus* and *S. nigrum* ranged from 9.33 to 42.45 mg GAE/g fw and 9.43 to 23.88 mg GAE/g fw, respectively. The findings indicate a higher phenol content in *P. fraternus* and a lower content in *S. nigrum* compared to the values reported by Butkute *et al*. [25] for the leaves of *A. glycyphyllos* (25.99 mg GAE/g dw). *P. fraternus* fruits and *S. nigrum* leaves both had higher phenolic contents than the corresponding fruit and leaf samples. The presence of phenols in the *P. fraternus* and *S. nigrum* extracts provided preliminary indication of their potential antioxidant activity, which increased as content of both plant extracts increased. Polyphenols can perform electron-donation reactions with oxidizing chemicals, resulting in stable species, and preventing or delaying the oxidation of many biomolecules [26]. As a result, plant phenols like Vitamin E (-tocopherol) have antioxidant characteristics. The presence of phenolic antioxidants, which are powerful free radical terminators, is a good indicator of prospective antioxidant action. According to Chang *et al.* [27], gallic acid, -coumaric acid, and caffeic acid were the primary phenolic components present in the *S. nigrum* extract. The phenolic hydroxyl groups of phenolic substances may explain their great ability to scavenge free radicals. Polyphenols have anticarcinogenic, antimutagenic, anticancer, antibacterial, antiviral, and anti-inflammatory properties by quenching ROS and inhibiting lipid peroxidation [28].

The total flavonoid content in the *P. fraternus* and *S. nigrum* was also determined by spectrophotometrically and calculated as QE ranged between 0.072 mg QE/g fw (Root) and 0.54 mg QE/g fw (Fruits) in *P. fraternus* and 0.032 mg QE/g fw (Root) and 0.33 mg QE/g fw (Leaf) in *S. nigrum,* respectively [Table 3]. Furthermore, the present study further reveals that total flavonoid content in the leaves extract of *P. fraternus* and *S. nigrum* (0.33-0.50 mg QE/g fw and 0.28-0.33 mg QE/g fw) in the present study is comparatively lower than the flavonoid content found in the extracts of leaves of *A. glycyphyllos* (21.00 mg RE/g), respectively [25]. Flavonoids are well-known plant compounds that function as antioxidants and have a variety of chemical and biological properties, including the capacity to scavenge free radicals. The flavonoids work by scavenging or chelating free radicals. Flavonoids have long been recognized for their potent antioxidant effects for human health benefits and fitness. Direct comparison of present data with the findings in literature may not be appropriate due to the variations in species, age, plant part, and the heterogeneity of the plant samples, etc. [Table 4]. In general, it can be inferred that the distribution of total phenolic content and total flavonoid content is influenced by the characteristics of the ecosystem. The variability in the results of the present study may be attributed to several factors, including ecological conditions, climate, genotypic variations, and environmental stress within different geographical locations where the herbal samples were collected [29].

In DPPH, ABTS, and FRAP assays, antioxidant properties in both plants were discovered in methanol extracts from different parts of the examined plants [Figure 6]. The antioxidants convert DPPH, a purple-colored stable free radical, into the colorless α-α-diphenyl-

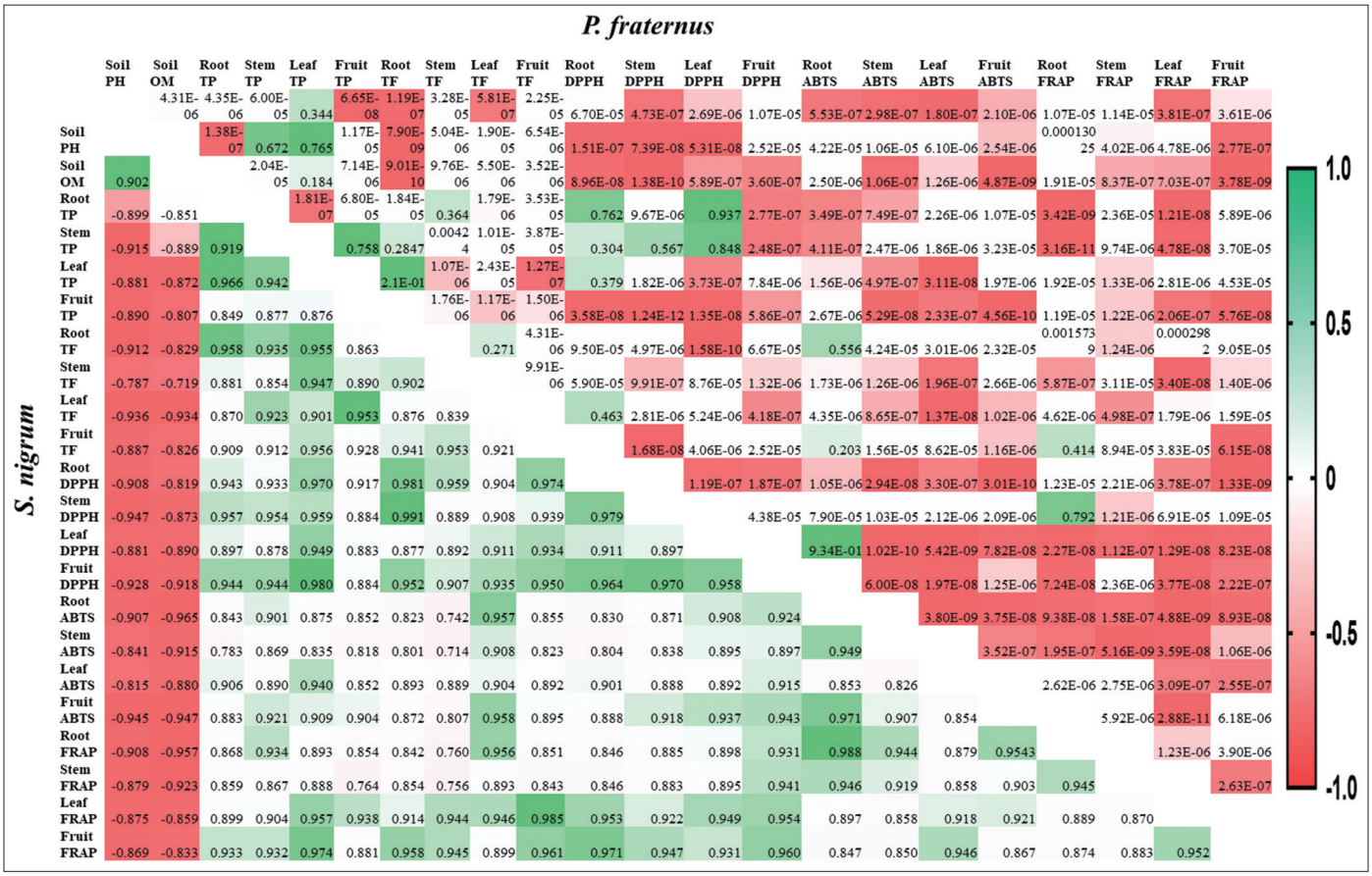

Figure 4: The Pearson correlation coefficient values are displayed as a heatmap in the correlation matrix, with positive values shown in green and negative values in red for each parameter under study. The scale runs from −1 to 1, with −1 denoting a perfect negative linear relationship between variables, 1 denoting a perfect positive linear relationship between variables, and 0 denoting no relationship between the variables under study. Plant biochemical characteristics of *P. fraternus* and *S. nigrum*, as well as soil physiological characteristics, were the study variables.

OM: Organic matter, TP: Total phenolics, TF: Total flavonoids.

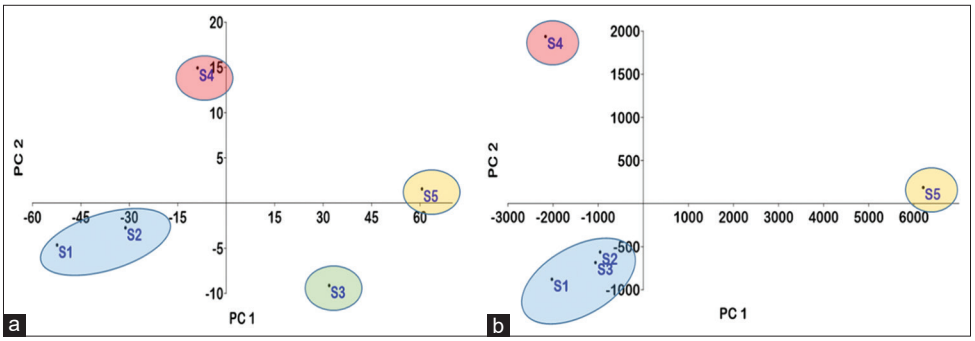

Figure 5: Principal component analysis of sampling location of *P. fraternus* and *S. nigrum* plants based on biochemical attributes and metals showing in (a) and (b), respectively.

α-picryl hydrazine. The decrease in absorbance at 517 nm could be utilized for the determination of the amount of DPPH that has undergone reduction [30]. The DPPH radical scavenging experiment was employed to measure the overall antioxidant activity of plant extracts from *P. fraternus* and *S. nigrum* in methanol [31] described the antioxidant properties of *S. nigrum* L. and discovered that the DPPH radical was inhibited by 54.16%. A similar result was reported by Harish and Shivanandappa [32]; in methanol extracts of *P. amarus* leaves and fruits inhibited membrane lipid peroxidation (LPO), scavenged 1,1-diphenyl-2picrylhydrazyl (DPPH) radical, and inhibited ROS *in vitro*, demonstrating the plant's antioxidant potential. Based on present findings, the observed peak activity in leaves using the DPPH technique aligns with the findings of Bronislava *et al.* [25], which indicated that *Astragalus cicer* leaves had significantly high antioxidant activity (128.6 µmol/g) in their leaf extract. In addition, the half-maximum inhibitory concentration observed in the ABTS assay of flowers exhibited comparable properties to the methanolic extract derived from flowers of *A. membranaceus* var. *mongholicus*

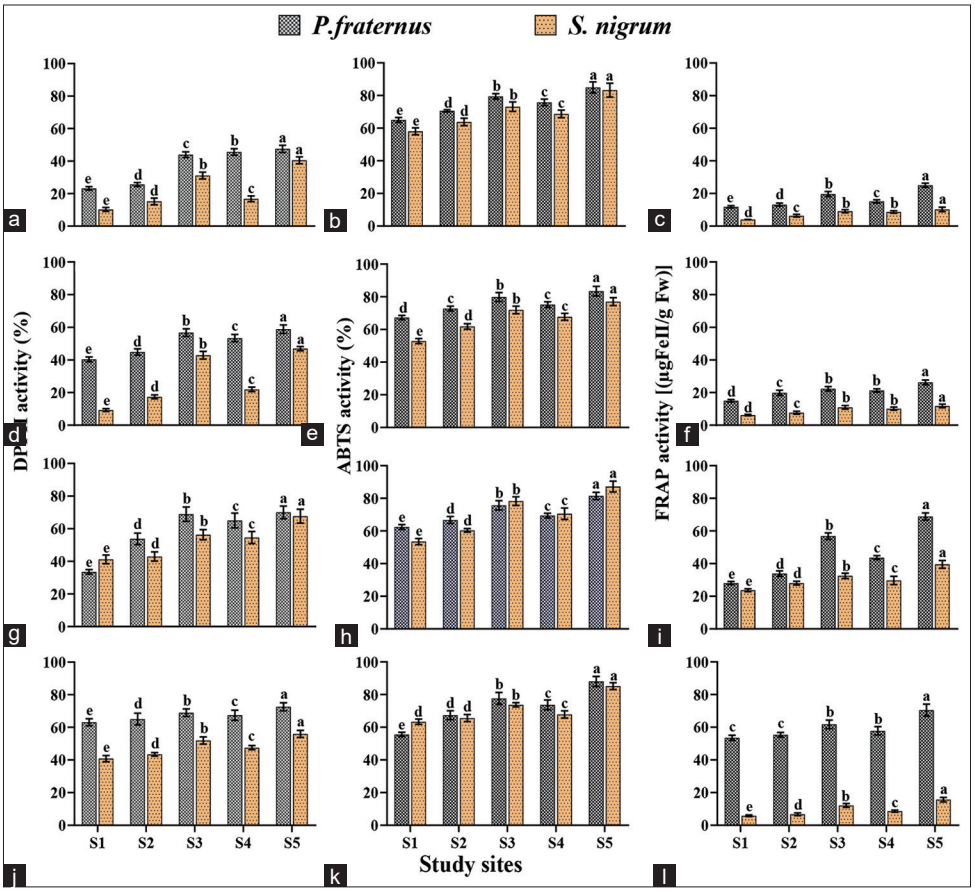

Figure 6: Antioxidant activities in methanol extract of root (a-c), stem (d-f), leaf (g-i), and fruit (j-l) of *P. fraternus* and *S. nigrum* plants growing in different areas of Varanasi. Bars are mean ± SE of three replicates. Bars adhered with different alphabets are statistically significant at $P < 0.05$ (Duncan's Multiple Range Test).

(22.02 µg/mL) as reported by Li *et al.* [33]. On the contrary, the seeds of *A. cicer* exhibited a significant antioxidant capacity, as shown by a 67.2% inhibition, which can be attributed to the presence and varying concentrations of antioxidant metabolites found in the respective medicinal plants [25]. Depending on the antioxidant activity and concentration, adding antioxidants to the pre-formed ABTS•+ radical cation reduces it to ABTS. Antioxidant molecules lower the blue-green color of the resulting solution and change its decolorization, and this is related to the amount of antioxidants in the plant extract [34]. The hydrogen-donating and chain-breaking antioxidant activity of plant extracts is commonly evaluated using the ABTS scavenging test [35]. This study exhibited the prospective capability of root, stem, leaf, and fruit extracts from *P. fraternus* and *S. nigrum* to scavenge ABTS free radicals. The scavenging activity escalated with higher concentrations of the sample extracts. The pharmacological effects of this plant extract include antimutagenic, antioxidant, and antiviral properties [36].

The FRAP assay assesses antioxidants' ability to reduce metals by electron donation. The results of the FRAP assay for determining antioxidant capacity are reported in [Figure 6]. The FRAP test is commonly used to evaluate antioxidant components in dietary polyphenols. Antioxidant activity rises as polyphenol concentration rises. Li *et al.* [37], recently found that the presence of total phenolics in numerous plants was associated with high FRAP values.

The principal component analysis was utilized for determining the relationships between the study sites located in Varanasi based on the biochemical characteristics of *P. fraternus* and *S. nigrum* as well as the heavy metal pollution index. The PC analyses showed that S1 and S2 are closely related to each other while S5 is negatively related to others studied sites. Plants collected from S5 sites are biochemically different from all other studied sites. PC analysis based on heavy metal pollution index which is represented by PC 1 (89.60 %) and PC (9.70 %) represents that S5 sites have negative correlation with S1, S2, and S3 [Figure 5b]. With the help of PCA analysis, it also can be depicted that S5 site stands differently from all other studied sites. It also depicted that slight increase level of metal in soil significantly increased the biochemical efficacy of *P. fraternus* and *S. nigrum* found in that particular area possibly to increase the cop up potential of plant to their adverse effects. Our findings, which are consistent with other studies, suggest that variation in TP and TF among populations is not related to geographic distance, but can be related to their natural environments [38].

The correlations between soil pH, soil organic matter, and antioxidant potential were examined using Pearson's correlation analysis [Figure 4]. In case of *S. nigrum* plant, a strong positive correlation was detected between root TF with stem DPPH ($R^2 = 0.99$) as well as between root TF with root DPPH ($R^2 = 0.98$). Bramorski *et al.* [39] found a similar association between antioxidant activity and total phenolics or total flavonoids. The minimum positive correlation was found between stem TF with stem ABTS ($R^2 = 0.75$). Similar positive correlation was found among TP–DPPH [40]. There was

Table 4: F ratio and level of significance of three-way ANOVA test for biochemical attributes of *P. fraternus* and *S. nigrum* plants collected from different study sites located in the Varanasi region of Indo-Gangetic plains of India.

Parameters	Variables						
	PS	PP	S	PS×PP	PS×S	PP×S	PS×PP×S
Total phenolics	5994.4***	3822.8***	625.7**	3066.7***	3067.0**	129.93**	60.86***
Total flavonoids	7702.6***	3815.0***	270.3***	922.8***	922.8**	7.21***	11.64***
DPPH	8903.0***	6346.5***	2948.3***	517.8*	517.8***	114.46***	87.47***
ABTS	74.4***	1048.6*	438.2***	32.3***	32.3***	14.31**	7.80***
FRAP	15932.4**	5948.2***	677.6**	3736.5**	3736.0***	89.90*	43.39***

PS: Plant species, PP: Plant parts, S: Sites, *P. fraternus*: *Phyllanthus fraternus, S. nigrum: Solanum nigrum,* Levels of significance: *p < 0.05, **p < 0.01, ***p < 0.001

found strong negative correlation between soil PH and soil organic matter with other variables of all biochemical attributes of *S. nigrum*. Soil organic matter showed a strong negative correlation with stem TF (R^2 = −0.79) and weak correlation with root ABTS (R^2 = −0.97). In the soil, PH strong negative correlation was found with stem TF (R^2 = −0.79) and weak correlation with stem DPPH (R^2 = −0.95). Furthermore, in case of *P. fraternus*, Pearson correlation revealed that there was found strong negative correlation between root TF with stem DPPH (R^2 = 1.24 E-12), These findings were consistent with those obtained by Wang *et al.* [41] who reported a negative correlation between DPPH-scavenging activity and TF of *S. tschiliensis* extracts and weak negative correlation was found between soil PH with fruit DPPH (R^2 = 1.07 E-05). Stem TP showed strong positive correlation with leaf DPPH (R^2 = 0.937), Similar positive correlation was found among TP–DPPH [42] although leaf TP have found poor positive correlation with root TP (R^2 = 0.184). Additionally, Bibi *et al.* [43] showed significant positive and negative correlations between antioxidant activities, TPC and TFC in bulbs of tested onion (*Allium cepa* L.). Relationship between FC-ABTS, TFC-DPPH, TPC-DPPH, and TPC-FRAP were found highly significant and positive (P < 0.05). Ni, Cd, Cu, and Zn are the most common heavy metals derived from anthropogenic sources found in agricultural soils [44]. As a result, radar plots were used to visually compare the concentrations of these elements across sampling sites, as shown in Figure 2.

A regression analysis was performed between the soil MPI and the biochemical activities of *P. fraternus* and *S. nigrum* root, stem, leaf, and fruit [Figure 3]. The R^2 value has been used to assess the relationship between soil MPI and biochemical activities. In terms of total phenolics, *S. nigrum* leaf (R^2 = 0.90) and root (R^2 = 0.77), rather than *P. fraternus* leaf (R^2 = 0.86) and fruit (R^2 = 0.75), had the highest and lowest R^2 values, respectively. The maximum and lowest R^2 values for total flavonoids were found in the leaves of *P. fraternus* (R^2 = 0.89) and the stem (R^2 = 0.71) rather than the fruits and roots of *S. nigrum* (R^2 = 0.86) and (R^2 = 0.80), respectively. The maximum and minimum R^2 values for DPPH, ABTS, and FRAP were found to be in *P. fraternus* fruit (R^2 = 0.92), root (R^2 = 0.72), leaf (R^2 = 0.91), and fruit (R^2 = 0.84), leaf (R^2 = 0.90), and stem (R^2 = 0.86), respectively, rather than *S. nigrum* leaf (R^2 = 0.92), root (R^2 = 0.85), and fruit (R^2 = 0.90), respectively. Bose *et al.* [45] reported similar outcomes as well.

In the present study, correlation between soil MPI and biochemical activities was statistically significant (P < 0.001). According to [46], the high correlation indicates that the phenolic and flavonoid contents significantly contributed to the antioxidant activity. The current analysis demonstrates a significant relationship between the tested samples antioxidant activity and their total phenolic and flavonoid content. [46]. Plants under heavy metal stress often experience increased levels of oxidative stress due to the generation of reactive oxygen species [47]. To counteract this oxidative stress, plants activate their antioxidant defense systems, which include enzymes such as superoxide dismutase, catalase, and non-enzymatic antioxidants like glutathione and ascorbic acid. The relationships between heavy metal exposure and the upregulation of antioxidant mechanisms in plants are of great significance. Phenolics and flavonoids produced as secondary metabolites in plants also function as reducing agents by donating electrons and interacting with free radicals to form more stable products under stress condition [48]. The present study further revealed that in *P. fraternus,* the maximum total phenolic, total flavonoid, ABTS, and FRAP were found in leaf except DPPH in fruits. But, in case of *S. nigrum,* total phenolic, DPPH, and FRAP were maximally reported in leaf except total flavonoid and ABTS in fruits [Figure 6]. The variations in antioxidant properties of the methanol extracts in leaves, fruit, stem, and roots of the tested plant species may also be ascribed to the level of heavy metal contamination in the soil of the study areas.

5. CONCLUSION

The findings of the present study indicate that both *P. fraternus* and *S. nigrum,* specifically their roots, stems, leaves, and fruits, are rich sources of total phenolics and flavonoids. Furthermore, these plant parts exhibit significant antioxidant activities as measured by DPPH, ABTS, and FRAP assays. The present research revealed the potential of *P. fraternus* fruits and *S. nigrum* L. leaves as valuable reservoirs of natural antioxidants. Natural antioxidants hold promise for mitigating or delaying the onset of age-related ailments associated with oxidative stress, such as hypertension, arthritis, atherosclerosis, and hepatic toxicity. These plants have a long history of traditional use in tropical and subtropical regions for various health issues, and the presence of abundant polyphenols such as phenolics and flavonoids adds to their medicinal value. The present findings not only provide insight into the therapeutic potential of these medicinal herbs but also offer a foundation for standardizing herbal medications, potentially playing a significant role in modern medicine.

6. AUTHORS' CONTRIBUTION

Umesh Kumar conducted the experiments, performed all the sample analyses, and prepared first draft of the manuscript. Indrajeet Kumar, Prince Kumar Singh, Jay Shankar Yadav, Akanksha Dwivedi, Priyanka Singh, and Saumya Mishra assisted in the analysis and prediction of experimental data and preparation of figures/tables. Rajesh Kumar Sharma developed the concept of the present study and performed the final editing of the manuscript. All authors have reviewed and approved the final version of the manuscript for publication.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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