Original Article

Deciphering the *In Vitro* Antioxidant Potential and Mineral Analysis of *Fagopyrum* Species from Kashmir and Ladakh Regions

Fayaz Ahmad Dar, Tanveer Bilal Pirzadah, Inayatullah Tahir[1], Reiaz Ul Rehman

Department of Bioresources, University of Kashmir, Srinagar, Jammu and Kashmir, India, [1]Department of Botany, University of Kashmir, Srinagar, Jammu and Kashmir, India

Abstract

Context: The Kashmir and Ladakh Himalayan regions are having a rich diversity of buckwheat germplasm, which is an excellent source of nutrition and functional food. The objective of this study was based on comparative *in vitro* flavonoid, antioxidant, and mineral analyses of *Fagopyrum* species grown in these regions. **Materials and Methods:** To achieve this goal, leaf samples from the four buckwheat species were subjected to antioxidant analysis. Besides, the mineral analysis of the groat samples of different buckwheat species was carried out by atomic absorption spectroscopy (AAS). **Results:** Results indicated that the methanolic extract shows higher total phenolic content (TPC) and total flavonoid content (TFC) in the samples of *Fagopyrum sagittatum* followed by *Fagopyrum tataricum*, *Fagopyrum kashmirianum*, and *Fagopyrum esculentum*. Total reducing power (TRP), ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion radical (SOR), and hydrogen peroxide (H_2O_2) radical scavenging assays indicated excellent results from the leaf extracts of *F. sagittatum*. The results suggested that the crude methanolic extract of buckwheat species had effective reducing power, radical scavenging activity, and metal-chelating ability compared to other standard antioxidants. AAS analysis revealed that calcium content was higher in *F. sagittatum* (21,600 ppm), whereas the iron and zinc contents were higher in *F. kashmirianum* (1,122.5 ppm) and *F. sagittatum* (166.75 ppm), respectively. **Conclusion:** Our study suggested that methanolic extracts of *Fagopyrum* species could act as a potent source of natural antioxidants to the pharmaceutical and food industry. In addition, the study also revealed that the rich elemental profiles of buckwheat species specify their therapeutic value and thus could be used as a potential biofortification crop.

Keywords: *1,1-diphenyl-2-picrylhydrazyl, antioxidants, atomic absorption spectroscopy, buckwheat, ferric reducing antioxidant power, functional food, hydrogen peroxide*

Introduction

Modern way of living has seriously affected our lives, especially our food preferences, and thus the concept of healthy diet has become one of the fundamental topics to deal with this problem. Buckwheat (*Fagopyrum* spp.), a dicot pseudocereal, is a potential candidate for nutraceutical and gluten-free cereal-based products pertinently due to high nutritional profile of the grain, especially for the patients with celiac disease. It has become an important food crop in many European and American countries as it consists of all the essential nutrients besides having appreciable antioxidant properties.[1,2] The studies on animals and humans have shown several health benefits, and thus it is being promoted as the functional food. The essential bioactive constituents are flavonoids, phytosterols, fagopyrins, fagopyritols, phenolic compounds, resistant starch, dietary fiber, lignans, vitamins, minerals, and antioxidants. The proteins of buckwheat are of high nutritional quality because it constitutes a well-balanced amino acid composition unlike common cereals.[2-4] The genus *Fagopyrum* includes a number of species, predominantly *Fagopyrum esculentum*, *Fagopyrum sagittatum*, *Fagopyrum tataricum*, and *Fagopyrum kashmirianum*, which are widely distributed in the Kashmir and Ladakh regions and are used as a food and fodder crop. Epidemiological studies have revealed that consumption of buckwheat is very effective against chronic diseases, and such beneficial effects can be attributed to high levels of antioxidants, especially tocopherols and polyphenols.[5] Buckwheat grains have a higher antioxidant, phenolic, and flavonoid content than other cereal crops. The protein content of Tartary buckwheat is 38.2% higher than rice, 3.9% higher than wheat, 30.5% higher than corn, and 20.2% higher than

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Address for correspondence:
Dr. Reiaz Ul Rehman, Department of Bioresources, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India.
E-mail: rreiazbiores@gmail.com

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common buckwheat.[6] Buckwheat products have important medicinal properties that help in reducing blood cholesterol and blood sugar, and cure hypertension, arteriosclerosis, lung diseases, liver ailments, and backache.[2,7] Antioxidants have recently acquired increased attention as they help in countering the negative effects caused by the generation of free radicals and oxidative processes.[8] Most of the organisms, including humans, have antioxidant defense mechanisms for protection against oxidative stress. However, the inherent antioxidant defense mechanisms are not sufficient to meet the requirements, and therefore dietary intake of essential antioxidants has been recommended.[9] It has been suggested that fruits, vegetables, and certain crop plants are the important sources to meet the extra demands of antioxidants required by the body. Natural antioxidants include radical scavengers, reducing power agents, complexes of pro-oxidant metals, quenchers of singlet oxygen, and so on. From the past few decades, considerable interest has been generated toward natural antioxidants for their use in food and medicinal products, thereby replacing the synthetic antioxidants, because of their long-term adverse effects.[10,11] Therefore, pseudocereals, such as buckwheat, which is highly nutritious and antioxidative, are being promoted to be used in brewing and baking industry. Moreover, the antioxidant activity in buckwheat is comparatively higher than oats, barley, wheat, rye, and most fruits and vegetables.[12]

The important phenolic and flavonoid compounds present in buckwheat grains and leaves are rutin, quercetin, quercitrin, isoquercitrin, hyperin, quercitrin 3-*O*-rubinoside, and kaempferol-3-*O*-rutinoside. Besides, it also consists of C-glycosyl flavones such as vitexin, isovitexin, orientin, and isoorientin. The concentration of phenolic and flavonoid compounds varies with the type of species, variety, and growth conditions.[13-15] Buckwheat is also an important source of minerals indispensable for human health.[16] However, in the current scenario, the quality of the agricultural land has been degraded because of malpractices and continuous use of synthetic fertilizers,[17] which in turn leads to the accumulation of obnoxious mineral elements in the plant system. Furthermore, there is scarcity of information regarding mineral analysis in buckwheat. Bonafaccia *et al.*[18] reported cobalt (Co)/antimony (Sb)/barium (Ba)/selenium (Se)/silver (Ag)/mercury (Hg)/chromium (Cr)/rubidium (Rb)/zinc (Zn)/iron (Fe)/nickel (Ni), and tin (Sn) contents in the flour and bran of buckwheat, where most minerals are concentrated mainly in the bran. Mestek *et al.*[19] also reported that the extract of buckwheat flour was significantly enriched with molybdenum (Mo)/nickel (Ni)/phosphorus (P)/cobalt (Co)/copper (Cu)/iron (Fe)/zinc (Zn), and manganese (Mn) mineral elements.

In view of the aforementioned facts, this study was undertaken to evaluate the antioxidant potential and mineral analysis of various buckwheat species (*F. esculentum* Moench, *F. sagittatum* Gilib, *F. tataricum* Gaertn, and *F. kashmirianum* Munshi) grown in Kashmir and Ladakh regions.

Materials and Methods

Plant material

Seeds of four buckwheat species (*F. esculentum*, *F. sagitatum*, *F. tataricum*, and *F. kashmirianum*) were collected from different sites (Gurez, Kargil, and Leh districts) of Kashmir and Ladakh regions as shown in Table 1, and were identified and deposited under the voucher no. 1978-KASH, in the Centre for Diversity and Taxonomy, Department of Botany, University of Kashmir, Hazratbal, Srinagar. Later on in the month of April 2016, fresh and healthy seeds of the buckwheat species were sown in the pots containing soil and sand in the ratio of 1:1 under the uniform set of light and temperature. The leaf material was harvested from 14-day-old plantlets. For atomic absorption spectroscopy (AAS) analysis, few seeds of each buckwheat species were sown during the month of April 2016 in the Kashmir University Botanical Garden (KUBG). Harvesting of the fresh seed samples was done at the fully mature stage.

Collection and preparation of sample material

Fresh and healthy leaves from the buckwheat species (*F. esculentum*, *F. sagittatum*, *F. tataricum*, and *F. kashmirianum*) were collected and washed gently with distilled water (without squeezing) to remove debris and dust particles. The plant material was then air-dried under shade at room temperature for 15 days, and ground into a powdered form using a surface sterilized mortar and pestle and then stored in plastic containers for extraction. For AAS, fresh seed samples were dried at 55°C for 72 h, mechanically grinded, and sifted out with a mesh (178 µm).

Preparation of solvent extract

Leaf extract was prepared using methanol as solvent, following the methodology of Okogun[20] with minor modifications. Methanolic leaf extract of *F. esculentum*, *F. sagittatum*, *F. tataricum*, and *F. kashmirianum* was prepared by mixing 5 g of dried fine powder in 50 mL of 80% methanol, and was constantly agitated on a rotary shaker (200 rpm, 25°C, 48 h). Extract was then filtered through Whatman's filter paper no. 1, and the filtrate was centrifuged (8000 rpm, 12°C, 15 min) to get clear methanolic phase. The final concentration of 10 mg/mL stock solution was prepared through dilutions, and the crude extract of each species was stored at 4°C for further analysis.

Table 1: Collection of buckwheat species from different sites of Kashmir and Ladakh regions

S. no.	Location	Latitude (N)	Longitude (E)	Altitude (amsl) m
1.	Izmarg, Gurez, Kashmir	34°658'	74°683'	2395
2.	Shelikchey, Kargil, Ladakh	34°586'	76°117'	2446
3.	Achithang, Leh, Ladakh	34°506'	76°626'	2820
4.	Khaltsi, Leh, Ladakh	34°326'	76°881'	2999

Determination of total phenolic content

Total phenolic content (TPC) was calculated by Folin–Ciocalteau (FC) reagent following the method of Mallick and Singh.[21] Briefly, 0.1 mL of methanolic extracts of different species was mixed with 0.5 mL of 1 N FC reagent, and the final volume was made up to 1 mL by the addition of double distilled water. The mixture was kept for 5 min at room temperature, followed by the addition of 2 mL of 20% sodium carbonate (Na_2CO_3) solution and mixed thoroughly. The final volume was made up to 5 mL with double distilled water, boiled for 1 min, and allowed to cool down. The absorbance was measured at 650 nm using double beam ultraviolet–visible (UV-VIS) spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). A gallic acid standard (R^2 = 0.998) was used to determine the TPC.

Determination of total flavonoid content

Total flavonoid content (TFC) was estimated by following the method of Hung and Morita[22] with slight modifications. In this assay, 0.1 mL of methanolic leaf extracts of different plant species was mixed with 0.4 mL of double distilled water and 0.5 mL of 0.1 M aluminum chloride ($AlCl_3$), 2 mL of 1 M potassium acetate (CH_3COOK), and 2 mL of double distilled water, and the final volume was made to 5 mL. After incubating the mixture at room temperature for 20 min, the absorbance was read at 415 nm using UV-VIS spectrophotometer (UV-1800, Shimadzu). A rutin standard (R^2 = 0.99) was used to determine the TFC.

Antioxidant assays

Total reducing power assay

Total reducing power (TRP) activity of the methanolic extracts was estimated by following the methodology proposed by Yen and Duh.[23] Various extracts (20, 40, and 80 µL) were mixed with 500 µL of 20 mM phosphate buffer (pH 6.6) and 500 µL of 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 20 min. Thereafter, 500 µL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 2500 rpm for 10 min. The upper aqueous phase was thoroughly mixed with 2.9 mL distilled water and 500 µL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. A stronger absorbance indicates stronger reducing power.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay was performed according to the protocol described by Benzie and Strain,[24] which is based on intense blue color development as the reaction involves the reduction of ferric iron (Fe^{3+}), TPTZ (2,4,6-tri (2-pyridyl)-1,3,5-triazine) complex to ferrous iron (Fe^{2+}) at low pH. Briefly, 30 mL of FRAP assay solution (consisting of 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution, and 20 mM ferric chloride solution). The mixture was incubated at 37°C for 10 min before use. Different concentrations of the plant extracts and standard (10 and 20 µL) were allowed to react with 2 mL of FRAP assay solution. The absorbance was measured at 593 nm at 37°C after 30 min of incubation. Calibration standard was linear between 200 and 1000 µmol $FeSO_4$, and the result was expressed in µmol Fe (II)/g dry weight (DW).

1,1-diphenyl-2-picrylhydrazyl radical scavenging assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was estimated according to the method described by Braca *et al.*[25] In this assay, 0.2 mM of the DPPH was prepared in absolute methanol. Various concentrations of plant extracts (20, 40, and 80 µL) were added to the DPPH solution, and the reaction mixture was thoroughly mixed and allowed to run in dark for 10 min. The absorbance (A) of the solution was measured at 517 nm using UV-VIS spectrophotometer (UV-1800, Shimadzu). The percentage inhibition of absorbance was determined for each dilution using the following equation:

$$\% \text{ inhibition} = \left[A_{\text{control}} - A_{\text{sample}} / A_{\text{control}} \right] \times 100$$

where A_{control} is absorbance value of the DPPH solution of the control and A_{sample} is absorbance value of the DPPH solution of the methanolic extracts of buckwheat species.

To determine IC50 value, that is, amount of sample required to cause 50% inhibition of DPPH radical, the scavenging percentage was plotted against logarithmic values of concentration, and a linear regression equation, $Y = mx + C$, was established.

Superoxide anion radical scavenging assay

Superoxide anion radical (SOR) scavenging activity of the methanolic leaf extracts was estimated following the methodology of Fontana *et al.*[26] with slight modifications. Briefly, 1.5 mL of 100 mM phosphate buffer (pH 7.4), 0.3 mL of 50 mM riboflavin, 0.25 mL of 20 mM phenazine methosulfate (PMS), and 0.1 mL of 0.5 mM nitro blue tetrazolium (NBT) were mixed, before the addition of different concentrations (10, 20, and 40 µL) of sample extracts. Mixture was illuminated in the fluorescent light for 20 min, and the absorbance of the mixture was recorded at 560 nm. The percentage inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = \left[A_0 - A_1 / A_0 \right] \times 100$$

where A_0 is absorbance of the control and A_1 is the absorbance of the sample.

To determine IC50 value, that is, amount of sample required to cause 50% inhibition of superoxide anion radical, the scavenging percentage was plotted against logarithmic values of concentration, and a linear regression equation, $Y = mx + C$, was established.

Hydrogen peroxide radical scavenging assay

Hydrogen peroxide (H_2O_2) radical scavenging activity was determined by following the modified protocol of Ebrahimzadeh *et al.*[27] Briefly, various methanolic extract concentrations (10,

20, and 30 µL) were added to 600 µL of 40 mM H_2O_2 solution and 0.1 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 25°C for 10 min, and the absorbance was recorded at 230 nm using UV-VIS spectrophotometer (UV-1800, Shimadzu). The H_2O_2 radical scavenging activity was calculated by using the following formula:

$$H_2O_2 \text{ scavenging activity } (\%) = [A_0 - A_1 / A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

To determine IC50 value, that is, the amount of sample required to cause 50% inhibition of H_2O_2 radical, the scavenging percentage was plotted against logarithmic values of concentration, and a linear regression equation, $Y = mx + C$, was established.

Mineral element analysis

Sample preparation for atomic absorption spectroscopy

Wet ashing was done following the protocol of Ang and Lee[28] by taking 0.25 g of powdered groat samples in a separate 50 mL flask containing mixed acid solution (nitric acid [HNO_3]:sulfuric acid [H_2SO_4]:perchloric acid [$HClO_4$]) in a ratio of 5:1:0.5. The samples were boiled in acid solution under fume hood on hot plate till the digestion was completed, indicated by white fumes coming out of the flask. Thereafter, few drops of ultrapure water were added and allowed to cool. The volume of the digestion solution was adjusted to 50 mL with ultrapure water. The solution was filtered and submitted to AAS (PerkinElmer Analyst 100, Waltham, Massachusetts) analysis.

Statistical analysis

Each experiment was performed in triplicate, and the results were expressed as mean ± standard deviation (SD). The IC50 was calculated using linear regression analysis. The Graphpad Prism 8 for Windows (Graphpad software La Jolla, California) was used for statistical analysis. Differences were considered to be significant at $P < 0.05$. In case of AAS, the data obtained were analyzed by correlation analysis, principle component analysis, and hierarchical cluster analysis to reveal the distribution rules of the elements.

Results

Total phenolic content and total flavonoid content

TPC and TFC of the 14-day old leaf samples of four buckwheat species (*F. esculentum*, *F. sagittatum*, *F. tataricum*, and *F. kashmirianum*) are presented in Figure 1A and B. The results clearly revealed that the methanolic extract of *F. sagittatum* shows higher TPC (145.92 ± 11.76 mg GAE/g DW) followed by *F. kashmirianum* (119.52 ± 8.29 mg GAE/g DW), *F. tataricum* (108.60 ± 6.74 mg GAE/g DW), and *F. esculentum* (81.18 ± 5.43 mg GAE/g DW). In the similar manner, the highest TFC was found in *F. sagittatum* (118.44 ± 8.06 mg RE/g DW) followed by *F. tataricum* (108.75 ± 6.21 mg RE/g DW), *F. kashmirianum* (93.29 ± 4.96 mg RE/g DW), and finally *F. esculentum* (73.68 ± 4.34 mg RE/g DW).

Total reducing power activity

TRP of the methanolic extract increased with the increase in the concentration of the extract in the buckwheat species. From this study, the reducing power of the methanolic leaf extracts among the four species of buckwheat was in the order of *F. sagittatum* > *F. tataricum* > *F. kashmirianum* > *F. esculentum* as shown in Figure 1C. In case of *F. esculentum*, the optical density (OD) of the methanolic extract increases from 0.270 ± 0.07 at 20 µL to 0.865 ± 0.14 at 80 µL concentrations. In *F. sagittatum*, the OD of the extract increases from 0.629 ± 0.12 at 20 µL to 1.401 ± 0.19 at 80 µL concentrations. In *F. tataricum*, the OD varies from 0.405 ± 0.09 at 20 µL to 1.201 ± 0.17 at 80 µL concentrations. Finally, in *F. kashmirianum*, the OD varies from 0.421 ± 0.10 at 20 µL to 1.151 ± 0.16 at 80 µL concentrations.

Ferric reducing antioxidant power activity

The reducing power of plant extracts, which is associated with antioxidant activity, was measured using the FRAP assay. FRAP is a simple and rapid method for screening the antioxidants in the number of plant species. Samples, which possessed antioxidant compounds, were able to reduce Fe (III) in potassium ferricyanide to Fe (II), which resulted in changing the solution color from yellow to light green. From this study, it was observed that the ferric reducing power of the *Fagopyrum* species increased in a concentration-dependent manner [Figure 1D]. Results reveal that the methanolic leaf extract of *F. sagittatum* shows better ferric reducing power (259.04 ± 14.56 µmol Fe (II)/g DW) at 20 µL concentration, followed by *F. tataricum* (162.6 ± 10.38 µmol Fe (II)/g DW), *F. kashmirianum* (125.83 ± 9.32 µmol Fe (II)/g DW), and *F. esculentum* (102.31 ± 8.87 µmol Fe (II)/g DW) over the same concentration.

1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

DPPH radical scavenging activity indicated that the extracts are capable of donating an electron or hydrogen atom to react with stable DPPH radical. From the results, it is clearly revealed that the radical scavenging activity of the leaf extract increases with the increase in the concentration of the extract. Among the four species of buckwheat, *F. sagittatum* shows highest scavenging activity (125.65 ± 13.38) at 80 µL concentration, followed by *F. tataricum* (105.54 ± 11.67), *F. kashmirianum* (96.15 ± 10.21), and the least activity was shown by the *F. esculentum* (81.54 ± 9.42) over the same concentration [Figure 1E].

Among the four species of buckwheat grown in the Kashmir and Ladakh regions, the highest DPPH activity was observed in the methanolic extract of *F. sagittatum*, whose IC50 value = 21.42 µg/mL, and the lowest DPPH activity was observed in *F. esculentum*, whose IC50 value was 42.86 µg/mL. The lowest IC50 value was linked with the highest DPPH activity [Table 2]. This study clearly indicates that

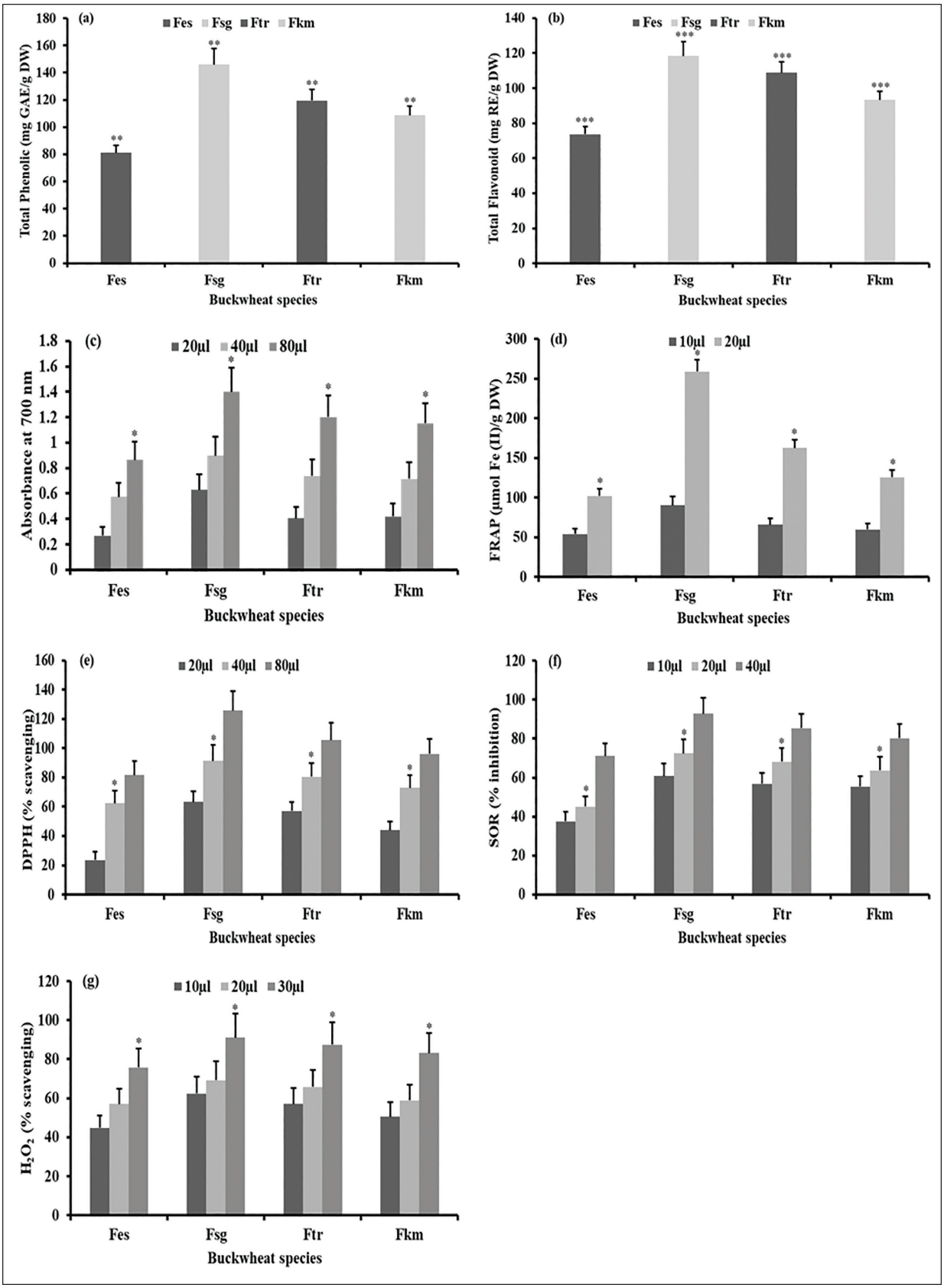

Figure 1: Concentration of (A) total phenolic (B) total flavonoid (C) total reducing power (D) ferrous reducing antioxidant power (E) DPPH radical scavenging activity (F) superoxide anion radical scavenging activity, and (G) hydrogen peroxide radical scavenging activity of the 80% methanolic leaf extracts of *F. esculentum* (Fes), *F. sagittatum* (Fsg), *F. tataricum* (Ftr), and *F. kashmirianum* (Fkm). Data represent mean ± SD (*n* = 3). *Significance at *P* < 0.05

methanolic extract of *F. sagittatum* is comparatively better in terms of radical scavenging activity among the four species of buckwheat grown in Kashmir and Ladakh regions.

Superoxide anion radical scavenging activity

Methanolic leaf extracts of buckwheat shows highest SOR scavenging activity in a dose-dependent manner [Figure 1F]. The highest SOR scavenging activity was found in *F. sagitattum* (92.78 ± 8.32) at 40 µL concentration, followed by *F. tataricum* (85.41 ± 7.48), *F. kashmirianum* (80.2 ± 7.26), and *F. esculentum* (71.07 ± 6.64) over the same concentration.

Among the four buckwheat species grown in Kashmir and Ladakh regions, the lowest IC50 value (IC50 = 14.35 µg/mL) was found in the methanolic extract of *F. sagittatum*, and the highest IC50 value (IC50 = 24.56 µg/mL) was found in the methanolic extract of *F. esculentum*, which suggests that the methanolic extract of *F. sagittatum* has more scavenging power of $O_2^{\bullet-}$ radicals compared to all other species of buckwheat grown in these regions [Table 2].

Hydrogen peroxide radical scavenging activity

From the results, the H_2O_2 scavenging activity of the methanolic leaf extracts of four buckwheat species increased in a dose-dependent manner to quench OH radicals and was found to be high in *F. sagitattum* (91.01 ± 8.78) at 30 µL concentration, followed by *F. tataricum* (87.48 ± 11.31), *F. kashmirianum* (83.15 ± 10.27), and *F. esculentum* (75.76 ± 9.82) over the same concentration [Figure 1G].

The IC50 value of methanolic extract was found to be IC50 = 12.98 µg/mL for *F. sagittatum* and IC50 = 17.18 µg/mL for *F. esculentum* [Table 2]. The higher antioxidant activity and lower IC50 value suggests that *F. sagittatum* has the highest peroxide radical scavenging activity among the four species of buckwheat grown in Kashmir and Ladakh regions. This may be due to the presence of several important bioactive compounds in the methanolic extracts such as phenols and flavonoids, which are capable of yielding protons or hydrogen atoms to stabilize free radicals.

Mineral element analysis

The important minerals found in all the buckwheat species are calcium (Ca) and Fe, which are present in abundant concentrations as shown in Figure 2. However, *F. sagittatum* contains highest Ca concentration (21,600 ± 76.4 parts per million [ppm]), followed by *F. tataricum* (5,125 ± 56.76 ppm), *F. esculentum* (4,402 ± 30.44 ppm), and *F. kashmirianum* (4,055 ± 45.67 ppm). The Fe concentration among four different species of buckwheat was found in the order of *F. kashmirianum* (1,122.5 ± 25.77 ppm) > *F. sagittatum* (1,065 ± 15.45 ppm) > *F. esculentum* (990 ± 12.45 ppm) > *F. tataricum* (875 ± 10.86 ppm). Slight difference was found in Mn concentration among *F. esculentum* (128.25 ± 13.65 ppm) and *F. kashmirianum* (127.27 ± 11.55 ppm). The results also revealed that the Zn concentration among different species of buckwheat was found in the order of *F. sagittatum* (166.75 ± 15.32 ppm) > *F. kashmirianum* (122.75 ± 12.34 ppm) > *F. tataricum* (104.25 ± 10.34 ppm) > *F. esculentum* (101 ± 9.89 ppm). Cu and Cd concentration was found in the range of 24–32 ppm and 13–17 ppm, respectively [Table 3]. Results also revealed that Ni, Cr, and Co concentration slightly varies between the four different species of buckwheat. The correlation analyses of 11 mineral elements are presented in Table 4. The positive and negative correlations among different elements are presented by the correlation coefficient (*R* value). The *R* value close to ±1 depicts strong positive or negative correlation. From the results, Cr shows a strong positive correlation with Al (R = 0.967). Similarly, Ni is highly correlated with Cd (R = 0.986).

Table 2: Fifty percent inhibitory concentration (IC50) of different plant extracts

	IC50 (µg/mL)		
Extracts	**DPPH$^{\bullet}$**	**SOR$^{\bullet}$**	**$H_2O_2^{\bullet}$**
F. esculentum	42.86	24.56	17.18
F. sagittatum	21.42	14.35	12.98
F. tataricum	26.23	16.31	14.04
F. kashmirianum	32.16	17.38	15.72

Discussion

Total phenolic content and total flavonoid content

The presence of phenolic and flavonoid compounds indicates that plant extracts potentially have antioxidant activities. From the present investigation, it was revealed that TPC and TFC were found to be higher in *F. sagittatum*, followed by *F. tataricum*, *F. kashmirianum*, and *F. esculentum* [Figure 1A and B]. A comparative study on two buckwheat species also revealed that TPC and TFC were higher in *F. tataricum* as compared to *F. esculentum*, using different solvents for extraction.[29,30] It has been found that rich phenolic and flavonoid contents in plants could be a vital source of therapeutic potential against the oxidative damages caused by free radicals, besides possessing other health-promoting benefits. This is the major reason that buckwheat has been found very effective in reducing the blood cholesterol level, arteriosclerosis, high blood pressure, and in keeping the capillaries and arteries strong and flexible.

Total reducing power activity

The reducing power activity of the compounds could serve as an important indicator of the antioxidant potential, and this property can be used to study the ability of the extracts to transform Fe^{3+} to Fe^{2+} by donating an electron.[31] The ability of the extracts to reduce Fe^{3+} could be attributed either to the reducing agents, such as phenolic groups, and the number and position of OH molecules on these groups.[32] The TRP of the methanolic extract increased with the increase in the concentration of the extract in the buckwheat species, and the maximum activity was observed in the *F. sagittatum* among the four species of buckwheat grown in Kashmir and Ladakh regions as shown in Figure 1C. The lowest activity

was observed in the *F. esculentum*, despite being most widely cultivated species of buckwheat in the Kashmir and Ladakh regions. Similar observation was made on two buckwheat species in which the reducing power activity was higher in *F. tataricum* as compared to *F. esculentum* using methanol as a solvent.[33] Many reports have suggested that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts.[34,35]

Ferric reducing antioxidant power activity

FRAP assay measures the reducing ability of an extract reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ), and has been used in a number of plants to determine antioxidant activity.[36] This study indicated that ferric ion reducing activities of *F. sagittatum* has been comparatively higher than other species of buckwheat (*F. tataricum*, *F. kashmirianum*, and *F. esculentum*) [Figure 1D]. Similar observation was also reported in *F. tataricum* and *F. kashmirianum* that show an increase in ferric reducing antioxidant power ability as the concentration increases,[37] and in *Crataegus* spp.[38] From this study, it can be deduced that the methanolic leaf extracts of four buckwheat species have variable abilities to scavenge free radicals, and therefore can act as a potent source of antioxidants.

1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

DPPH activity is considered as one of the most reliable assays for determining the free radical scavenging activities in most of the plant extracts.[39] Production of free radicals in the living systems is a natural process and is related to many disorders such as neurodegenerative diseases, aging, and cancer. The

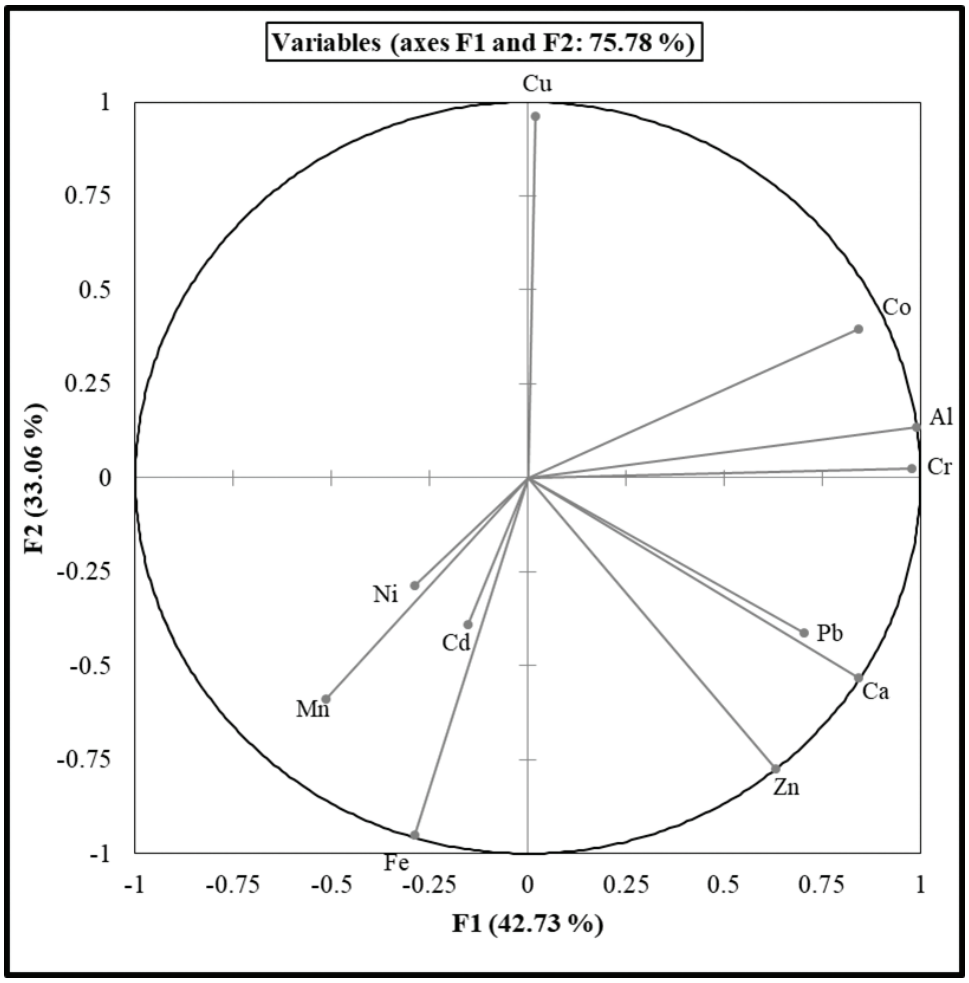

Figure 2: Principal component analysis (PCA) among four species of buckwheat grown in Kashmir and Ladakh regions

Table 3: Concentration of various mineral elements in the groat of four buckwheat species (ppm)

	Al	Cu	Fe	Mn	Cd	Ni	Ca	Pb	Zn	Co	Cr
Fes	572.5	31.25	990	128.25	13.25	0.25	4402	0.75	101	0.75	0.5
Fsg	692.5	25.25	1065	113.5	15.25	1	21600	1.25	166.75	1.5	2
Ftr	637.5	31.25	875	86.5	15.75	1.5	5125	0.25	104.25	1.75	1.5
Fkm	495	24.5	1122.5	127.27	16.75	2	4055	0.25	122.75	0.5	0.25

Table 4: Correlation matrix for the elemental concentrations in the various species of buckwheat

Variables	Al	Cu	Fe	Mn	Cd	Ni	Ca	Pb	Zn	Co	Cr
Al	1										
Cu	0.155	1									
Fe	-0.411	-0.886	1								
Mn	-0.576	-0.409	0.785	1							
Cd	-0.223	-0.621	0.299	-0.260	1						
Ni	-0.342	-0.527	0.241	-0.254	**0.986**	1					
Ca	0.763	-0.473	0.275	-0.062	-0.004	-0.171	1				
Pb	0.652	-0.230	0.263	0.244	-0.473	-0.612	0.864	1			
Zn	0.514	-0.752	0.550	0.100	0.256	0.094	0.937	0.730	1		
Co	0.882	0.299	-0.663	-0.893	0.048	-0.023	0.466	0.219	0.242	1	
Cr	0.967	-0.015	-0.331	-0.649	0.034	-0.093	0.789	0.553	0.605	0.911	1

Values in bold are different from 0 with a significance level $\alpha = 0.05$

free radical scavenging activity of antioxidants synthesized in plants helps in the management of these diseases and their detoxification. DPPH, being a stable free radical, can accept an electron or free radical to become a stable diamagnetic molecule.[40] Therefore, the methanolic extracts from the *Fagopyrum* species inhibited the DPPH radicals in a variable proportion depending on the species under study.[41] This difference can be attributed to the unequal distribution of the antioxidant molecules, especially phenols and flavonoids present in the buckwheat species. These extracts are composed of several scavenging compounds that work in a synergistic manner to enhance the antiradical activity. Moreover, the antiradical activity of the extracts to trap DPPH free radicals depends on the availability and the ability of these extracts to give electrons or hydrogen atoms.[42] The present investigation revealed that the DPPH free radical scavenging activity of methanolic leaf extracts was found to be higher in *F. sagittatum* and lower in *F. esculentum* in a dose-dependent manner among the four species of buckwheat [Figure 1E]. This study has been further supported by the IC50 values, which was lowest in *F. sagittatum* (21.42 µg/mL) and highest in *F. esculentum* (42.86 µg/mL), indicating that *F. sagittatum* is having strong potential to scavenge DPPH free radicals as depicted in Table 2. Zielińska *et al.*[14] also reported highest DPPH radical scavenging activity in the aerial parts of *F. tataricum* than *F. esculentum*, while evaluating antioxidant capacity of these two plant species.

Superoxide anion radical scavenging activity

Superoxide anion is considered as a weak oxidant produced during various biological reactions and is highly toxic.[43] It is known as an initial radical and plays a significant role in the formation of other reactive oxygen species, such as hydroxyl radical (˙OH) or singlet oxygen ($^1O_2$), which induce oxidative damage in lipids, proteins, and deoxyribonucleic acid (DNA).[44] The antiradical activity against SOR scavenging activity (in a riboflavin light-dependent NBT chloride system) was determined in case of methanolic extracts of four buckwheat species (*F. esculentum*, *F. sagittatum*, *F. tataricum*, and *F. kashmirianum*). This study indicated $O_2^{\bullet-}$ radical scavenging has been highest in *F. sagittatum*, followed *by F. tataricum*, *F. kashmirianum*, and *F. esculentum* in a dose-dependent manner [Figure 1F]. Furthermore, the IC50 values have been found to be lowest in *F. sagittatum* (14.35 µg/mL) and highest in *F. esculentum* (24.56 µg/mL) among the four buckwheat species, signifying the potent $O_2^{\bullet-}$ radical scavenging activity in *F. sagittatum* as compared to other species of buckwheat in this study [Table 2]. Similar observations have been reported in methanolic leaf extracts of *F. tataricum*[45] and *Gymnema sylvestre*.[46] From this study, it can be inferred that the superoxide scavenging activity of methanolic extract of different buckwheat species has the potential to scavenge superoxide anions. Moreover, the superoxide anion radical scavenging activity may be due to the action of free hydroxyl groups present on phenolic compounds.

Hydrogen peroxide radical scavenging activity

The OH radical formation through Fenton's reaction is an indication of events in the cells of living systems that causes *in vivo* cell damage such as lipid peroxidation, which ultimately leads to DNA mutagenesis and inactivation of various proteins.[11] The formation of OH radicals produced endogenously during aerobic metabolism in the living systems is considered by far the most predominant reactive oxygen species and extremely harmful as they cause inflammation, carcinogenesis, and toxicity of the tissues.[47] Therefore, the antioxidant capacity of a compound could be best determined by measuring the inhibition of OH radicals. From the results, it was revealed that the methanolic extracts caused hydroxyl radical scavenging in a concentration-dependent manner, which has been found to be highest in *F. sagittatum*, followed by *F. tataricum*, *F. kashmirianum*, and *F. esculentum* [Figure 1G]. The study also indicated that the methanolic extract of *F. sagittatum* showed higher, and *F. esculentum* showed lower H_2O_2 radical scavenging activity, which has been further supported by the low IC50 value of *F. sagittatum* (12.98 µg/mL) as compared to high IC50 value of *F. esculentum* (17.18 µg/mL) among the four buckwheat species [Table 2]. Sowndhararajan and Kang[10] also suggested that methanolic leaf extracts of *Bauhinia vahlii* possess significant OH radical scavenging activity among the various tested solvents. Zieliński and Kozłowska[48] reported that 80% methanolic extract produced

64 times more phenolic compounds and four times higher antioxidant activity than aqueous extract in buckwheat.

Mineral element analysis

Macro- and micronutrients play a significant role in the biological systems as these nutrients take part in various metabolic processes such as nerve function and energy metabolism.[49] Ca plays a significant role in muscular contraction, provides strength to bones, and reduces the risks of osteoporosis.[50] In our study, Ca was found to be high in groat samples of all buckwheat species [Figure 2]. Fe, Cu, Cr, Zn, and Mn possess numerous cellular functions. Fe constitutes an important part of the hemoglobin, thus is necessary to overcome the problems of anemia, besides it also maintains the function of central nervous system (CNS).[51,52] Fe is also important to prevent cough linked with angiotensin-converting enzyme inhibitors. From the results, buckwheat groat was found to be rich in Fe content, and the highest concentration was found in *F. kashmirianum* (1122.5 ± 25.77 ppm). Cu also takes part in various metabolisms, and the deficiency of this mineral element leads to microcytic anemia, neutropenia, and deformation of bones.[53] Results reveal that the groat samples of different buckwheat species show a slight difference in the Cu concentration. Cr concentration was found high in *F. sagittatum* (2 ± 0.13 ppm). Cr plays a vital role in regulating blood glucose level, hunger, cholesterol level, and also protects DNA.[50] This study shows that the micronutrient Zn was found to be high in *F. sagittatum* (166.75 ± 15.32 ppm). Zn acts as a cofactor in various enzymes. Deficiency of Zn, especially in children, leads to growth retardation, loss of appetite, general indisposition, and skin-related disorders.[54] A groat sample of *F. esculentum* contains high levels of Mn (128.25 ± 13.65 ppm) as compared to rest of species. Mn is very essential micronutrient to improve insulin sensitivity, and it is the structural component of many enzymes.[55] Human body requires Co and Ni in a very little amount. Co is an essential component of vitamin B_{12} and thyroid metabolism.[56] Ni, Pb, Cd, and Al are considered as toxic elements and their presence in the buckwheat groat samples is due to the degraded quality of the soil. The concentration data were subjected to common chemometric analyses, including correlation analysis (CA) and principal component analysis (PCA), to gain better understanding of the differences among the tested samples [Figure 2]. Our results indicated that the essential mineral concentrations were not different between *F. tataricum* (L.) Gaertn and *F. esculentum* Moench. Similar results were revealed while studying the mineral contents of two buckwheat cultivars (Hajnalka and Oberon) grown in Hungary in which its seeds contain higher K, Mg, and Fe contents.[57]

Conclusion

From this study, it is concluded that *F. sagittatum* possesses the higher quantities of both phenolic and flavonoid compounds compared to the other species of buckwheat found in the Kashmir and Ladakh regions. *F. sagittatum* possesses highest antioxidant and antiradical activity, and *F. esculentum* possesses the lowest among the four species of buckwheat. The order of antioxidant and antiradical activity is as follows: *F. sagittatum* > *F. tataricum* > *F. kashmirianum* > *F. esculentum*. Methanolic extract shows higher activity, thus could be the optimal solvent for the extraction of bioactive constituents. It can also be concluded that a rich concentration of phenolic and flavonoid compounds in buckwheat is the major contributor to its high antioxidant potential. In addition, 11 macro- and micronutrients were analyzed in the groat samples of different buckwheat species grown in Kashmir and Ladakh regions. Results depict that buckwheat species have accumulated major mineral elements that possess an immense role in therapeutics; thus, they could be used as a potential biofortified crop.

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Conflicts of interest

There are no conflicts of interest.

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