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Antioxidant Potential of Common Leafy Vegetables in Eastern Zone of Nepal

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ABSTRACT

This paper is projected to cram the antioxidant activities the common leafy vegetables in eastern development zone of Nepal. The sprouts, microgreens and leafy greens of common and tartary buckwheat of Nepalese strains were compared for the phenolic contents and biological activity. The tartary buckwheat samples expressed higher total phenolic and flavonoid contents compared to the common buckwheat. The sprouts had the highest total phenolic contents (9333.48 ± 150.23 and 6976.21 ± 213.65 mgGAE/100g dw in tartary and common buckwheat, respectively) whereas, the highest total flavonoid content was present in the leafy greens (7635.39 ± 141.40 and 4414.61 ± 70.85 mg RE/100g dw in tartary and common buckwheat respectively). The high performance liquid chromatography (HPLC) results revealed that the tartary buckwheat vegetables had higher rutin, (3800.28 ± 434.41 mg/100g in leafy greens), quercetin (159.75 ± 9.04 mg/100g in sprouts) and chlorogenic acid (293.47 ± 65.06 mg/100g in microgreens) contents than those of common buckwheat. However, other phenolics like vitexin, isovitexin, orientin and isoorientin contents were more abundant in common buckwheat. In biochemical assay, all three types of vegetable of common and tartary buckwheat showed higher antioxidant and α-glucosidase inhibition effect in dose dependent manner. Based on these results, it can be conformed that all the vegetables (microgreens, sprouts and leafy greens) of both varieties of buckwheat of Nepalese strains can be regarded as a potent sour ce of functional food.They also displayed high total antioxidant capacity. Therefore, the top five potential leafy vegetables consist of both hydrophilic and lipophilic antioxidant(s), the order being I. aquatica> B. campestris> B. alba> P. sativum> and L. siceraria carried out .

1. Introduction

The Eastern Development Region consists of 3 zones (Koshi, Mechi and Sagarmatha) and 16 districts. Dhankuta is the headquarters of the development region. The area of Eastern Development Region is 28,546 square kilometer. According to the Census 2001, its population is 5,344,476. and the density is 187.815/km2 (486.440/sq mi).In this region most of the people are using green vegetables so now-a-days, antioxidants are considered as important as vitamins for promotion of health and prevention of various diseases linked to reactive oxygen species (ROS). ROS have been linked to over 100 disorders [1]. Therefore, for maintaining a healthy biological system, it is critical to have the balance between oxidation and antioxidation. Excess generation of ROS causes oxidative stress that damage DNA, lipids and proteins of cells leading to pathogenesis of various diseases such as cardiovascular disorders, diabetes, cancer, inflammation, aging, brain dysfunction, etc. A variety of phytochemicals have been reported as showing antioxidant potential [2] The majority of antioxidants are hydrophilic and they can easily contribute to keep up physiological health of hydrophilic organs whether lipophilic antioxidants are essential for lipophilic organs such as brain, cell membrane and skin. Leafy vegetables are popular in eastern zone of Nepal but there is no scientific data available on the content of functional components in it. So the present research was undertaken to study the polyphenol, flavonoid, anthocyanin and antioxidant content of the leafy vegetables of Eastern Zone of Nepal. Leafy vegetables are widely consumed in many countries because of their nutritional quality and anthocyanin, ascorbic acid, β-carotene, flavonoid, folic acid, polyphenol and alkaloid contents. These components have a wide range of biological functions such as antiallergic, anticancer, antidiabetic, antimicrobial, antioxidant, anticardiovascular [3]. Reportedly, various epidemiological studies have suggested that consumption of fruits and vegetables is associated with reduced risk of aging, inflammation, cancer, cardiovascular diseases, Alzheimer's and Parkinson's. Nowadays, antioxidants are considered as important as vitamins for promotion of health and prevention of various diseases linked to reactive oxygen species (ROS). ROS have been linked to over 100 disorders [4] Therefore, for maintaining a healthy biological system, it is critical to have the balance between oxidation and antioxidation. Excess generation of ROS causes oxidative stress that damage DNA, lipids and proteins of cells leading to pathogenesis of various diseases such as cardiovascular disorders, diabetes, cancer, inflammation, aging, brain dysfunction, etc. A variety of phytochemicals have been reported as showing antioxidant potential [5].The majority of antioxidants are hydrophilic and they can easily contribute to keep up physiological health of hydrophilic organs whether lipophilic antioxidants are essential for

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lipophilic organs such as brain, cell membrane andskin. Leafy vegetables are popular in Bangladesh but there is no scientific data available on the content of functional components in it. So the present research was undertaken to study the polyphenol, flavonoid, anthocyanin and antioxidant content of the leafy vegetables of eastern zone of Nepal. Reactive oxygen species (ROS), such as superoxide radical, hydroxyl radical, hydrogen peroxide, and singlet oxygen are associated with different diseases like cancer, aging, cardiovascular diseases, inflammation and neurodegenerative diseases [6]Likewise, diabetes is a metabolic disorder disease that occurs worldwide and its incidences increasing rapidly in most parts of the world. In modern medicine no satisfactory effective therapy is still available to cure the diabetes mellitus. Hence, there has been increasing interest in using medicinal plants to control diabetes and ROS mediated diseases. Buckwheat is a plant that possesses both antioxidant and antidiabetic properties which is attributed to its phenolic contents like rutin and [7]. This plant is considered as a functional food as it is rich in phenolic compounds including rutin, quercetin, orientin, vitexin, isovitexein and isoorientin[9]. Among these compounds rutin, a flavonol glycoside has been recognized as a major antioxidant component that accounts for about 85-90% ofthe total antioxidant activity [10].Rutinis also known to have anti-inflammatory,anti-carcinogenic and is effective for preventing hemorrhagic disease and arteriosclerosis . Likewise, quercetin (aglycone), a major bioflavonoid of human diet, present in buckwheat, has been identified as a strong antioxidant, anti-angiogenesis and anticancer .It is also known to reduce the risk of hypertension .Besides rutin and quercetin, the other flavonoids like vitexin, isovitexin, orientin and isoorientin are also considered as good antioxidant compounds present in buckwheat (Kim et al., 2008) and have been reported to exhibit 4-40% of antioxidant activities (Szostak, 2004).Vitex in has been found to be effective in the prevention of skin cell damage caused by UV- radiation .. Likewise, potential antimicrobial and antifungal activity have been shown by isovitexin (Morris, 2003) and chlorogenic acid .Orientin has the ability to protect radiation-induced lipid peroxidation in mouse liver[11] and isoorientin is known to scavenge free radicals and prevent human LDL (low density lipoprotein) against oxidation [12].Nowadays, buckwheat sprout and microgreens have gained popularity due to the functional compounds present in them and are considered as a new vegetable(Kim et al., 2001). So far, several researches have beenreported regarding the phenolic content and antioxidant activities of the buckwheat sprouts [13]. However, the results were variedin different researches. This variation could be due to the fact that the buckwheat phenolics can be influenced by geographic origin of seed as well as environmental conditions. Likewise, phenolics or flavonoids (rutin) content in buckwheat can also be influenced by solar radiation photoperiods and cultivation time [13].In context to Nepal, buckwheat is an important crop ofthe hilly area, and is a staple food crop in the remote hills. Apart from the grains, the young shoots (3 to 4 weeksold) of the buckwheat plant are also generally consumed as leafy vegetable by the people of Himalayan region. Ina previous study, high rutin content in different parts of buckwheat that originate from Nepal has been reported [14]). However, there are no reports regarding the phenolic compositions and bioactivity of the Nepalese strain of buckwheat sprout, micro greens and leafy greens. Hence, the objective of this study was to compare the three kinds of vegetables viz. sprouts, microgreens and leafy greens of common and tartary buckwheat, originating from the eastern hills of Nepal for their phenolic contents and other biological (antioxidant and α-glucosidase inhibition) properties (common buckwheat) and *Fagopyrumtartaricum*(tartary buckwheat) were collected from local market of Illam hills and different domestic market of Eastern Development Zone of Nepal. The sprouts of common and tartary buckwheat were grown in a dark chamber at a temperature of 25°C, watered from time to time and were harvested at 7 days. The sprouts were dried at temperature of 50°C, crushed and stored in the refrigerator for further experiments.

Cultivation of buckwheat microgreens and leafy greens

The seeds of *F. tartaricum* and *F. esculentum* were sown in an open field in natural environment and watered from time to time (one time in a day till 7 days and alternate day till 3 weeks). On the7 days and 21 days of germination, the young plants were harvested as microgreens and leafy greens, respectively. The harvested samples were washed and the roots were removed before drying at a temperature of 50°C.

Preparation of plant extracts

The dried powdered samples (2 g) of both varieties of buckwheat were taken and 100 ml of 80% ethanol was added to each and incubated overnight in a shaker followed by filtration using Advantech 5B Tokyo Roshi Kaisha, Japan. The extract was dried using a rotatory evaporator (Eyela N-1000, Japan) at temperature of 40°C. The extracts were vacuum freeze dried to remove the remaining moisture. The yield was measured and stored in the refrigerator for further experiment.

2. Materials And Methods

The leafy vegetables of Amaranthusgangeticus L., Amaranthusviridis L., Basella alba L.,Brassica campestris L., Centellaasiatica L., Chenopodium album L., Coriandrumsativum L.,EnhydrafluctuansLour., Ipomoea aquaticaForssk., Lagenariasiceraria (Mol.) Standl., Pisumsativum L. and Spinaciaoleracea L. were collected from the local markets of Eastern Zone of Nepal. After washing with distilled water the collected vegetables were shed dried. The dried sample was ground to powder by grinding machine and stored in air tight containers. Ethanol and pentane were used as solvents. Twenty five grams of powder of each sample was taken in 100 ml of ethanol and kept in airtight bottle. After 7 days, the ethanol was filtered by Whatman No. 1 filter paper. The filtrate wasair dried and the solid extracts were kept in the refrigerator. For the preparation of lipophilic extract, 10 g powder was shaken vigorously by hand with 200 ml pentane and after filtration the filtrate was air dried to obtain pentane extract. Finally 10 mg of the solid extract was dissolved in1 ml ethanol or DMSO (10 mg/ml) and used to conduct the experiments. The total concentration of phenolics (TPH) in ethanol extracts was determined (in triplicate)according to the Folin-Ciocalteu method with gallic acid (GA) as the standard and expressed (mg) as gallic acid equivalents (GAE)/g of extract .Total flavonoids and anthocyanins contents were determined according procedure with slight modification .The reaction mixture (total volume, 3 ml), consisting of 0.5 ml of 0.5 M acetic acid buffer solution at pH 5.5, 1 ml of 0.2 mM DPPH in ethanol, and 1.5 ml of 50% (v/v) ethanol aqueous solution, was shaken vigorously with the ethanol or pentane extracts (Blois 1958). After incubation at room temperature for 30 min, the amount of DPPH remaining was determined by measuring absorbance at 517 nm.

Mean values were obtained from triplicate experiments. The scavenging effect of ethanol extracts on nitric oxide was measured after Marcocci*et al.* (1994), while the reducing power was determined following Oyaizu (1986). Total antioxidant capacity was measured according to Prieto *et al.* (1999).Results are expressed as mean ± SD for a given number of observations (n = 3 - 5). The level of significance was set at *p* value of 0.05.

Plant materials and sprout production Buckwheat sample of two varieties Fagopyrumesculent

2.1. Estimation of total polyphenol and flavonoid content

The total phenolic (TP) content was determined by the Folin-Ciocalteu assay (Eom et al., 2008). A sample aliquot of 200 µl was added to a test tube containing 200 µl of phenol reagent (1 M). The volume was increased by adding 1.8 ml of distilled deionized water and the solution was allowed to stand for 3 minutes for reaction after vortex. Further to continue reaction, 400 µl of Na2CO3 (10%, v/v) was added and vortexed and the final volume (4 ml) was adjusted by adding 1.4 ml of distilled demonized water. A reagent blank was prepared using distilled deionized water. The absorbance was measured at 725 nm after incubation for 1 h at room temperature. The TP content was expressed as Gallic acid equivalents (GAE) inmg/100g (dw) of sample. The total flavonoid (TF) content was determined according to Eom et al. (2008) with slight modifications. Briefly, an aliquot of 0.5ml of sample (1 mg/ml) was mixed with 0.1 ml of 10% aluminum nitrate and 0.1 ml of potassium acetate (1 M). In the mixture, 3.3 mlof 80% methanol was added to make the total volume 4 ml. The mixture was vortexed and the absorbance was measured after 40min at 415 nm in spectrophotometer and calculated. Rutin was used as a standard and the values of TF content were expressed in rutin equivalent (RE) mg/100g (dw).

2.2. HPLC analysis

The quantitative estimation of different compounds (rutin, quercetin,vitexin, isovitexin, orientin, isoorientin and chlorogenic acid) were performed by HPLC. The HPLC system (CBM-20A, Shimadzu Co,Ltd., Japan) with two gradient pumps (LC-20AT, Shimadzu), an auto sample injector (SIL-20A, Shimadzu), a UV-detector (SPD-10A,Shimadzu) and a column oven (35°C CTO-20A, Shimadzu) were used for analysis. The separation was performed on a C18 column (Synergi 4 µ MAX-RY, 150 × 4.6 mm, 4 micron Phenomenex). Flow rate of mobile phage solution was 1.0 ml/min, and detection was at355 nm. 10 µl of each sample was injected in to the HPLC machine

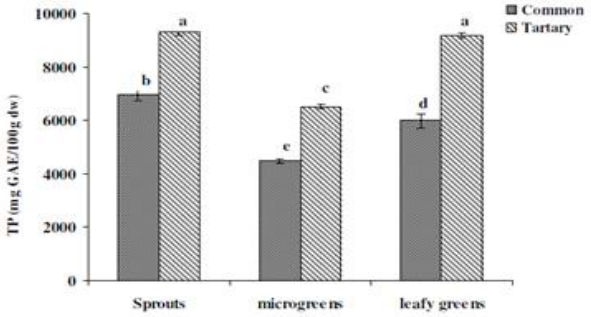

Figure 1. Total polyphenol (TP) contents in the 80% ethanolic extract of sprouts, microgreens and leafy greens of common and tartary buckwheat. TP contents are expressed in Gallic acid equivalent (GAE) in mg/100 g dw of sample. Each value is expressed as the mean ± SD (n=3). Different letters indicate that the values are significantly different (*P≤0.05*).

HPLC conditions were as follows: Solvent A (water in 0.1% Trifloroacetic acid) and solvent B (acetonitrile). Gradient elution used was 0-10 min, 5-6% B; 10-15 min, 6-10% B; 15-45 min, 10- 19% B; 45-65 min, 19-20% B.

2.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavengingactivity

The antioxidant activity of the samples (sprout, microgreens and leafy greens) was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described with slight modifications. Briefly, 1 ml of each of the extracts at different concentrations (0.125, 0.25, 0.5 and 1 mg/ml) was added to 4 ml (0.15 mM DPPH solution) of DPPH. The mixtures were shaken vigorously and left to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm in a spectrophotometer (Hitachi U-2001, Japan) and the percent inhibition activities of the extracts were calculated against a blank using the following expression: Inhibition (%) = (1-B/A) x 100, where, A is the absorbance of the mixture without extract and B is the absorbance of the mixture containing the extract of buckwheat vegetables.

2.4. Metal chelating power

The samples were analyzed for the metal chelating activity according to the procedure of Dinis et al. (1994) with slight modification. Briefly, 0.5 ml of the sample extracts at different concentrations (0.25, 0.5, 1 and 2 mg/ml) were mixed with 0.1 ml of1 mM FeCl2 followed by the addition of 0.2 ml of 5 mM ferrozine,vortexed and kept for 10 min. For blank, the sample extracts was replaced by 80% ethanol. The absorbance of the mixtures was measured against the blank at 562 nm after the addition of 3.2 ml 80%ethanol.

2.5. α-Glucosidase inhibitory activity

α-Glucosidase inhibitory assay was performed according to Kim et al. (2004). 100 µl of 5 mMp NPG (p nitrophenyl α-D-glucoside) in 0.2 M sodium phosphate buffer (pH 6.8) was added as a substrate to the mixture of 50 µl of α-glucosidase (0.15 unit/ml) and 50 µl of sample to start the reaction. The reaction was conducted at 37°C for 15 min and stopped by the addition of 300 µl of 0.1 M Na2CO3. α-glucosidase activity was assessed by measuring the release of p-nitrophenol from pNPG at 405 nm. All tests were performed in independent triplicate (n=3) and data were expressed as mean ±SD.

2.6. Statistical analysis

All data were expressed as mean value ± standard deviation of the number of experiments (n=3) using Microsoft EXCEL program. Differences between the mean values of the multiple groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple tests using the SPSS 16.0 Inc., USA package. Statistical significance was considered at *P≤0.05*.

3. Results and Discussion

Total polyphenol (TP) and total flavonoid (TF) content of sprouts, microgreens and leafy greens

The TP contents in the ethanolic extracts of buckwheat sprouts, microgreens and leafy greens were determined from regression equation of calibration curve and expressed in gallic acid equivalent (mg GAE /100g) of dry plant material (Figure 1). The results reveal that the TP content of tartary buckwheat was comparatively higher than the common buckwheat in all the three types of vegetables studied in the research. In both types of buckwheat, the content of TP was in the order: sprouts >leafy greens >microgreens. Among all, the highest content of TP was found in the sprouts and leafy greens of tartary buckwheat (9333.48 ± 150.23 and 9194.19 ±113.97 mg/100g, respectively).

The TF content of the sprouts of tartary and common buck wheat were expressed in rutin equivalent (mg RE/100g dw) of dry plant material (Figure 2). The TF content was also higher in tartary buckwheat vegetables compared to the common type. The TF in the leafy greens of both types of buckwheat were higher (7635.39± 141.40 mg/100g in tartary and 4414.61 ± 70.85mg/100g in common buckwheat) compared to the sprouts and microgreens. The TF content in both types of buckwheat was in the order, leafy greens > sprouts >microgreens.

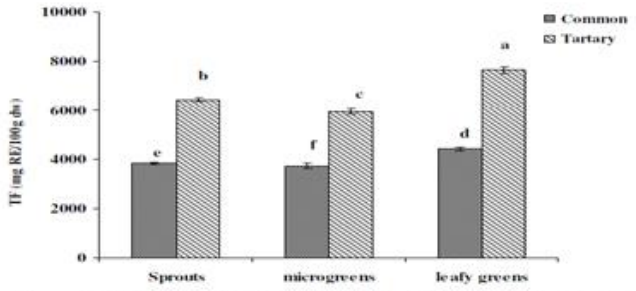

Figure 2. Total flavonoid (TF) contents in the 80% ethanolic extract of sprouts, microgreens and leafy greens of common and tartary buckwheat. Flavonoid content was expressed in rutin equivalent (RE) in mg/100 g dw of sample. Each value is expressed as the mean ± SD (n=3). Different letters indicate that the values are significantly different ($P \leq 0.05$).

Table1. Different phenolic contents in 80% ethanolic extract of sprouts, microgreens determined br HPLC.

Compound (mg/100g dw)		Common buckwheat
	Sprouts	Micrigreens
Rutin	384.42±36.43	595.81±65.10
Vitexin	581.27±21.92	394.15±43.32
Isovitexin	370.14±17.22	247.14±21.05
Orientin	352.25±28.47	208.56±8.02
Isoorientin	751.53±31.72	431.24±53.29
Quercrtin	8.52±3.14	4.74±0.22
Chlorogenic acid	27.21±6.70	156.23±2.04

Each value expressed as the mean ± SD (n=3)

Table 2. Different phenolic contents in 80% % ethanolic extract of sprouts, microgreens and leafy greens of tartary buckwheat determined by HPLC.

Compound (mg/100g dw		Tartary Buckwheat	
	Sprouts	Microgreens	Leafy greens
Rutin	3100.98±202.80	3000.12±343.12	3800.28±434.41
Vitexin	40.12±7.07	26.01±8.04	4.64±1.87
Isovitexin	18.44±8.11	11.73±6.75	3.01±0.02
Orientin	85.15±4.27	ND	ND
Isoorientin	152.65±11.43	85.10±5.42	ND
Quercrtin	159.75±9.04	7.13±2.02	171.43±2.02
Chlorogenic acid	41.26±6.31	296.47±65.06	51.55±6.32

Each value is expressed as the mean ± SD (n=3) No: Not detected

The variability in the flavonoid content and the antioxidative activity of buckwheat due to cultivar, cultivation time and location was reported by Oomah and Mazza, (1996). Park et al. (2004) also reported the significant variation in the rutin content in the tartar buckwheat strains collected from different regions.According to the data (Tables 1and 2), vitexin, isovitexin, orientin, and isoorientin seemed to decrease gradually in the microgreens and the leafy greens, suggesting that growth in the light or soil (as in microgreens) or longer growth (from microgreens to leafy greens) might have decreased the level of these compounds. While the higher level of rutin accumulated in the leafy greens compared to the sprouts and microgreens implies that longer growth in the presence of light might increase the rutin contents in buck wheat vegetables. In the present study, it was observed that the antioxidant activities such as, DPPH free radical scavenging activity and the metal chelating ability of the tartary buckwheat vegetables (sprouts, microgreens and leafy greens) were higher than the vegetables of common buckwheat. This higher antioxidant activity was due to the higher amount of rutin, quercetin and chlorogenic acid content in the tartary buckwheat . Ourresult support previous finding of Liu et al. (2008) where they also reported that the sprouts of tartary buck wheat scavenged higher percent of free radicals compared to those of the common buckwheat. However, the concentration required for scavenging approximately 88 % of DPPH radicals in their experiment was 5 mg/mL. Whereas, according to our data, the IC50 value for free radical scavenging activity of tartary sprout was 150.11ppm (Table 3), which was approximately 90 % at 1 mg/ml(percent data not shown). All this dissimilarity in the research could be due to the difference in phenolic compositions in different cultivar, growing season and location, soil types, harvesting times and other environmental. In the metal chelating assay, the microgreens of tartary buckwheat expressed the highest metal chelating activity with an IC50 value of 150.29 ppm. This may be due to the presence of high level of chlorogenic acid (Table 2), as it has been previously reported that a melandion-like polymer derived from chlorogenic acid was the main metal chelating substance in coffee. In the α-glucosidase inhibition assay, the sprouts were the best inhibitors of the enzyme with an IC50 of 44.56 and 78.36ppm for tartary and common buckwheat respectively.

4. Conclusions

In ours study, we observed that the TP and TF content of the sprouts, microgreens and leafy greens of tartary buckwheat were varied and comparatively higher than those of the common variety. This variation in the TP and TF content among the vegetables could be due to the fact that exposure to natural light and the age of the buckwheat sprouts affects the phenolic and flavonoid composition of buckwheat .In case of tartary buckwheat, the contents of some compounds like rutin, orientin, isoorientin and quercetin were much higher in our study, whereas, vitexin, isovitexin were lower compared to the sprouts reported by Kim et al. (2008). It was also noticeable in our research that the chlorogenic acid content in the microgreens was much higher (293.47 ± 65.06) compared tothe sprouts (41.26 ± 6.31 mg/100g dw). This is in accordance with previous research by Kim et al., (2008),Sharma et al. 189where they also found similar trend showing the microgreens having twice the amount of chlorogenic acid compared to the sprouts. In conclusion, all the vegetables (microgreens, sprouts and leafy greens) of both varieties of buckwheat can be regarded as a potent source of phenolics (rutin, quercetin, vitexin, isovitexin, orientin isoorientin and chlorogenic acids) and has high antioxidant activities.

Table 3. IC_{50} of DPPH free radical scavenging activity, metal chelating activity and a- glucoside inhibitory activity expressed ppm.

Analysis	Sprouts		Microgreens		Leafy greens	
	Common	Tartary	Common	Tartary	Common	Tartary
DPPH free radical scavenging activity	219.08	150.11	239.68	152.51	187.23	127.44
Metal chelating activity	839.05	572.88	291.82	150.29	290.79	260.06
a- glucoside inhibitory activity	78.36	44.56	6086.65	1532.33	874.93	323.22

In an in vitro antidiabetic assay using the enzyme α-glucosidase conformed that both the mentioned species of buckwheat vegetables can be esteemed as a potent inhibitor of α-glucosidase activity which can contribute in the treatment of diabetes. Overall, through this research, it is suggested that Nepalese strain buckwheat vegetables contain high phenolics with higher biological (antioxidant and α-glucosidase inhibition) activity and can be used as an alternative food. Therefore, mass production of more and more buckwheat food products should be encouraged and included in the daily diet, which would help the people to prevent diabetes and many other diseases caused by the free radicals.

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