



Antioxidant Potential of Selected Wild Edible Leafy Vegetables of Sikkim Himalayan Region: Effects of Cooking Methods and Gastrointestinal Digestion on Activity

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Green leafy vegetables or GLVs are one of the main attractions in the local vegetable market and are widely consumed as the main course and side dish in the Sikkim Himalayan region (SHR). This study evaluated the total phenolic (TPC) and flavonoid contents (TFC) and antioxidant potential in different extracts such as methanolic (MeOH), ethyl acetate (EtOAc), and hexane extracts of selected GLVs followed by changes in the antioxidant activity on cooking and stimulated gastrointestinal (GI) digestion. The MeOH extracts of *Urtica dioica* L. (Sisnu), *Nasturtium officinale* W. T. Aiton (Simrayo), *Diplazium esculentum* Retz. Sw. (Ningro), and *Chenopodium album* L. (Bethu) were estimated to have higher TPC [22.73–45.84 μg gallic acid equivalent (GAE)/mg of extract]. In contrast, the plant extracts prepared using EtOAc (except for *N. officinale*, where TFC was found to be higher in hexane extract) were found to contain higher TFC (3.42–14.86 μg quercetin equivalent (QE)/mg of extract). The MeOH extracts also exhibited higher 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (9.55–18.67 μg ascorbic acid equivalent (AAE)/mg of extract), total antioxidant activity (TAA) (0.27–0.32 mg AAE/mg of extract), and reducing power potential (RPP) (1.6–9.9 μg AAE/mg of extract). Among the test MeOH extracts, *U. dioica* demonstrated relatively higher antioxidant activities and was selected for cooking experiments followed by simulated GI digestion. The findings revealed that the loss of antioxidant activity was minimal in steam-cooked leaves (3.5% in 40 min) as compared to the boiled ones (18% in 10 min). The simulated GI (simulated salivary, gastric, and intestinal) digestion performed on raw, steam cooked, and boiled *U. dioica* leaves showed substantial enhancement of antioxidant properties (by 64.63%) through steam cooking in comparison to the raw leaves. Overall the study concludes that higher antioxidant properties can be achieved on the consumption of steam-cooked *U. dioica* leaves.

Keywords: green leafy vegetables, phenolic contents, antioxidant activity, *Urtica dioica*, Sikkim Himalaya

INTRODUCTION

Reactive oxygen species (ROS) which include free radicals such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (OH^-), singlet oxygen ($^1\text{O}_2$), and alkoxy radical (RO) are formed continuously in the human body because of different cellular metabolic processes (electron transport chain, phagocytosis, fertilization, energy production in mitochondria, etc.) (1–4). These ROS are reported to cause oxidative damage to living cells on reacting with different molecules of the cell and are responsible for the development of several pathological conditions including DNA damage, lipid peroxidation, protein oxidation, and cellular degeneration. Moreover, uncontrolled generation of ROS has also been associated with various diseases and disorders such as diabetes, cancer, rheumatoid arthritis, osteoporosis, and aging (1, 5). The human body has developed an endogenous defense system consisting of a few antioxidant enzymes (catalase, glutathione reductase, superoxide dismutase, etc.) to protect itself from the harmful effects of ROS; nevertheless, it fails to be effective against the oxidant load in certain conditions. Therefore, the human body needs to be supplied with exogenous antioxidants.

Antioxidants are small molecules that can help to limit or prevent the harmful effects of pervasive free radicals. These molecules are highly essential for the survival of all living beings. Plant-based foods are an excellent source of natural antioxidants such as flavonoids and related phenolic compounds that offer a balance between the oxidants and antioxidants in the body and thereby play role in combating the oxidative stress inside the human body (6). The green leafy vegetables or GLVs which are being commonly used in every household as a part of healthy and promising meals contain a blend of natural antioxidants and phenolic compounds that render them exceptional for several nutritional and important therapeutic properties (7–9). Studies have shown that regular consumption of GLVs rich in antioxidants results in a positive impact by decreasing oxidative damage (10). Conversely, before their consumption, most of the GLVs need to be undergone a cooking process (boiling, steaming, frying, etc.) which is based on taste preference and edibility. Cooking can also induce changes in the physical characteristics, the chemical composition of the food, and influence the concentration, release, and bioavailability of bioactive substances (11, 12). In certain cases, specific culinary methods have resulted in the denaturation of active constituents and thereby reducing the nutritional and therapeutic values. Specific cooking processes have also been shown to enhance the functional properties of selected GLVs (1, 13, 14). The extent of benefits anticipated from foods can also be influenced by the physiological process of digestion where the digestive enzymes act while the food travels through the gastrointestinal (GI) tract (13, 15).

Sikkim, a small Himalayan state of northeastern India, is known for its multiethnic population and notable biodiversity including subtropical and alpine climates. The wild edible leafy vegetables have been valued greatly throughout this region and serve as sources of food for the inhabitants. The mode of consumption and ethnopharmacological significance of some of

these vegetables have also been documented (16). Nonetheless, the data on the effects of different cooking processes on the bioactivity of such GLVs are still limited. In addition, the fates of bioactive components during the process of GI digestion have merely been studied. Therefore, this study was aimed at evaluating the antioxidant potential of organic extracts prepared from selected green leafy vegetables commonly used in the Sikkim Himalayan region (SHR), followed by investigating the effects of different cooking methods and *in-vitro* GI digestion on retention of their antioxidant properties. The outcome of the study may contribute to the promotion of consumption of GLVs in SHR and other regions of the country to use a selective cooking approach to assure better antioxidant benefits.

MATERIALS AND METHODS

Materials

Methanol (MeOH), ethyl acetate (EtOAc), hexane, sodium carbonate (Na_2CO_3), Folin–Ciocalteu reagent, aluminum chloride (AlCl_3), potassium acetate (CH_3COOK), sulfuric acid (H_2SO_4), sodium phosphate (Na_3PO_4), ammonium molybdate [$(\text{NH}_4)_2\text{MoO}_4$], 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide $\text{K}_4[\text{Fe}(\text{CN})_6]$, trichloroacetic acid (TCA), potassium chloride (KCl), sodium bicarbonate (NaHCO_3), potassium dihydrogen phosphate (KH_2PO_4), ammonium carbonate [$(\text{NH}_4)_2\text{CO}_3$], sodium chloride (NaCl), hydrochloric acid (HCl), magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), α -amylase, pepsin, pancreatin, calcium chloride (CaCl_2), and sodium hydroxide (NaOH) are from Sigma Aldrich.

Collection of Plant Samples and Preparation of Extracts

Green leafy vegetables (GLVs) used in this study including *Urtica dioica*, *Nasturtium officinale*, *Diplazium esculentum*, and *Chenopodium album* were purchased from Lal Bazaar, a local market under Gangtok Municipal Corporation, East Sikkim. Collected leaves were washed in running tap water, oven-dried (40°C), and powdered using a mixer grinder. A definite quantity of leaf powders was extracted with 1:5 (w/v) of three different organic solvents of varying polarity (methanol, ethyl acetate and hexane) using cold maceration. Briefly, the leaf powders (100 g) were dissolved differently in 500 ml of the organic solvent and shaken vigorously for 15 min. Then, the solutions were kept in dark and shaking was performed at every 12 h interval. After 72 h, the solutions were filtered using Whatman No. 1 filter paper and the solvents were made evaporated using Rota evaporator (R 100, Buchi) to obtain the organic extracts which were stored at 4°C for further use.

Estimation of Total Phenolic Content

Total phenolic content (TPC) of the organic extracts was estimated following the Folin–Ciocalteu method previously described by Rai et al. (17). Briefly, $50 \mu\text{l}$ (2.5 mg/ml) extract was mixed with 2 ml of 2% Na_2CO_3 followed by incubation at room temperature for 2 min. Thereafter, $100 \mu\text{l}$ of 50% Folin–Ciocalteu reagent was added to the reaction mixture and incubated at room temperature for 30 min. Absorbance was recorded at 720 nm

using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). All the analyses were performed in triplicates and data were expressed in terms of μg gallic acid equivalent (GAE)/mg of the organic extract.

Estimation of Total Flavonoid Contents

Total flavonoid contents (TFCs) of the organic extracts were determined following a standard protocol as described by Aryal et al. (18). To 1 ml (0.625 mg/ml) extract, 0.2 ml of 10% (w/v) AlCl_3 (in MeOH) and 0.2 ml (1 M) potassium acetate were added. The volume of the reaction mixture made up to 7 ml by adding distilled water and incubated at room temperature for 30 min. The absorbance was measured at 415 nm using a UV-VIS spectrophotometer. The data were expressed in terms of μg quercetin equivalent (QE)/mg of extract.

Determination of Total Antioxidant Activity

Total antioxidant activity (TAA) of the leaf extracts was determined following the method described by Rai et al. (19). Briefly, 200 μl (0.25 mg/ml) of extract was mixed with 3 ml reagent solution prepared using 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate (1:1:1). The components of the mixture were mixed well and incubated at 95°C in the water bath for 90 min. The reaction mixture was then allowed to cool to room temperature and the absorbance was recorded at 695 nm using a UV-VIS spectrophotometer. The data were expressed in mg ascorbic acid equivalent (AAE)/g of extract.

DPPH Radical Scavenging Activity

The leaf extracts were determined for their DPPH radical scavenging activity using the method described by Rai et al. (20). Briefly, 200 μl (1.25 mg/ml) extracts were mixed with 2 ml 0.16 mM DPPH solution followed by incubation in a dark condition for 30 min. After incubation, the absorbance of content was taken at 517 nm using a UV-VIS spectrophotometer. The DPPH scavenging activity was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \{1 - (S_{\text{abs}} - B_{\text{abs}}/C_{\text{abs}})\} \times 100$$

Where, S_{abs} —absorbance of sample, B_{abs} —absorbance of extract blank, and C_{abs} —absorbance of the control (DPPH).

Determination of Reducing Power Potential

Reducing power potential (RPP) was done according to the method as described by Rai et al. (20). Briefly, 100 μl (2.5 mg/ml) of the extract was mixed with 900 μl phosphate buffer (0.2 M) followed by the addition of 900 μl freshly prepared potassium ferricyanide (1%). The reaction mixture was vortexed and incubated at 50°C for 20 min. After the incubation, 900 μl of 10% TCA was added and centrifuged at 6,000 g for 10 min. The collected supernatant (900 μl) was added to an equal volume of distilled water and FeCl_3 (0.1%). Absorbance was taken at 700 nm using a UV-VIS spectrophotometer. The data were expressed in AAE/g of ascorbic acid.

Effects of Cooking on TPC, TFC, and Antioxidant Property of *U. Dioica*

The leaves of the *U. dioica* were cooked with two methods preferred by local people such as (i) boiling and (ii) steaming. Leaves of *U. dioica* (250 g) were divided into 5 equal parts. One part was taken as control and two parts were taken for boiling and the rest two were taken for steaming. Briefly, two sets of 50 g of leaves were taken in 400 ml distilled water and were boiled, respectively, for 5 min (B5) and 10 min (B10). Similarly, two sets of 50 g leaves were subjected to steam cooking for 20 min (S20) and 40 min (S40). The cooked leaves were then oven-dried at 40°C and ground to fine powders, which were then extracted in MeOH for further evaluation. The MeOH extracts were analyzed for TPC, TFC, and antioxidant activities (DPPH scavenging and TAA) as described earlier.

In-vitro Simulated Gastrointestinal Digestion of Cooked and Steamed *U. Dioica*

Simulation of GI digestion was performed *in-vitro* following standard protocol mentioned in Minekus et al. (21) with modifications wherever required. The boiled and steam-cooked *U. dioica* leaves were treated for sequential simulation of mouth, stomach, and small intestine digestion. The compositions of simulated salivary fluid (SSF), gastric fluid (SGF), and intestinal fluid (SIF) are given in **Table 1**. The oral digestion was done independently on 10 g of boiled and steam-cooked leaves which was mixed with 7 ml SSF followed by the addition of 1 ml α -amylase solution (1,500 U/ml in SSF), 50 μl CaCl_2 (0.3 M), and 1.95 ml distilled water to attain a paste of food: SSF-1:1 (w/v). The reaction mixture was then incubated for 5 min at room

TABLE 1 | Composition of simulated digestive fluids used in this study.

Simulated digestive fluids	Volume of chemicals used (in mL)					
	KCl (37.3 g/L)	KH_2PO_4 (68 g/L)	NaHCO_3 (84 g/L)	NaCl (117 g/L)	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (30.5 g/L)	$(\text{NH}_4)_2\text{CO}_3$ (48 g/L)
SSF, pH 7	30.2	7.4	13.6	-	1.0	0.12
SGF, pH 3	13.8	1.8	25.0	23.6	0.8	1.0
SIF, pH 7	13.6	1.6	85.0	19.2	2.2	-

SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

temperature. To the oral bolus 15 ml SGF, 3.2 ml pepsin solution (25,000 U/ml in SGF), 10 μ l CaCl₂ (0.3 M), and 1.39 ml distilled water were added for the simulation of the gastric digestion. The pH of the reaction mixture was maintained at 3 and incubated in shaking conditions at 37°C for 120 min. Similarly, simulation of the intestinal phase was performed on the gastric chyme with the addition of 22 ml SIF, 40 ml pancreatin solution (1:4 w/v), 80 μ l CaCl₂ (0.3 M), 0.3 ml NaOH, and 2.32 ml distilled water and then incubated at 37°C for 120 min at pH 7. After the complete simulation of digestion, the digesta were centrifuged at 4,000 rpm for 45 min and the supernatants were collected. The leftover residues were extracted with MeOH (1:5 w/v) to prepare the extract. TPC, TFC, and antioxidant activities were determined in both cases.

RESULTS AND DISCUSSION

Leafy vegetables are essential sources of minerals, such as microelements, namely, K, Ca, Mg, P, and S, and microelements, namely, Fe, Cu, Mn, Zn, Na, Mo, and B, protein, dietary fiber, carbohydrates, and vitamins for human nutrition (22–26). Most importantly, they are rich in natural antioxidants such as phenolics, betalains, xanthophylls, violaxanthin, ascorbic acids, carotenoids, betacyanins, betaxanthins, chlorophyll A, chlorophyll B, and beta-carotene that have high radical quenching ability (27–31). Phenolics, the nonnutrient secondary metabolites found in fruits, seeds, and vegetables, have long been known for their biochemical and pharmacological importance. Phenolic compounds are considered to be vital in defense responses of the human body including anti-inflammatory, antiaging, antiproliferative, and antioxidative mechanisms (32). They include coumarins, phenolic acids, such as hydroxybenzoic acids and hydroxycinnamic acids, flavonoids, such as flavonols, flavones, flavanols, flavanones, isoflavones, anthocyanins, chalcones, and nonflavonoids, such as tannins, lignans, and stilbenes (33–37). Among others, flavonoids are the most prevalent and ubiquitous group of phenolic compounds that are widely distributed in fruits, plant-derived beverages,

and vegetables. Mounting pieces of evidence in terms of epidemiological and clinical findings support their health-promoting and disease-preventing significance (38). Herein, selected GLVs consumed popularly in the SHR such as *U. dioica*, *N. officinale*, *D. esculentum*, and *C. album* (**Figure 1**) were studied for the determination of their TPC, TFC, and *in-vitro* antioxidant potential. The extract yield of MeOH, EtOAc, and hexane extracts of GLVs ranged from 3.97 to 8.05%, 1.77 to 4.50%, and 2.10 to 3.90% (dry weight basis), respectively (**Table 2**). Extract yield was found to be comparatively higher in MeOH extracts than that of EtOAc and hexane extracts. The TPC of GLV MeOH extracts ranged from 22.73 to 52.06 μ g GAE/mg, 10.16 to 32.76 μ g GAE/mg, and 9.14 to 25.40 μ g GAE/mg of extract, respectively. Similarly, TFC of GLV organic extracts ranged from 2.93 to 11.18 μ g QE/mg of extract in the MeOH, 3.42 to 14.86 μ g QE/mg of extract in EtOAc, and 1.87 to 10.84 μ g QE/mg of hexane extracts (**Table 3**). The *N. officinale* MeOH extract among others had the highest TPC (52.06 \pm 3.82 μ g GAE/mg of extract) followed by that of the *U. dioica*-MeOH extract (45.84 μ g GAE/mg of extract). The *C. album* hexane extract was found to have the lowest estimate of TPC (9.14 \pm 0.38 μ g GAE/mg of extract). The *U. dioica* EtOAc extract was found to have the highest TFC (14.86 μ g QE/mg of extract) followed by that of the MeOH (11.18 μ g QE/mg of extract) and hexane extracts (10.84 μ g QE/mg of extract). TFC was estimated to be the lowest in the hexane extract of *D. esculentum* (1.87 μ g QE/mg of extract). The overall results showed variation in the TPC and TFC among different organic extracts of the same GLV.

Biosynthesis and accumulation of phenolic compounds are appeared to be unconventional in each plant and plant organ, and variation in their contents relies on the growth stage and the genotypic composition of the plant species (39). The extractability and recovery of a particular organic component are reported to depend on the polarity of the extraction medium and the ratio of solute to solvent. Likewise, the extractability of phenolic compounds is reliant on the type and polarity index (PI) of the solvents used, and the solubility of the phenolic



FIGURE 1 | Selected green leafy vegetables popularly consumed in the Sikkim Himalayan region.

TABLE 2 | Scientific nomenclature and extract yield of selected leafy greens of Sikkim Himalayan Region.

Scientific names	Common names	Total yield (%)		
		MeOH	EtOAC	Hexane
<i>Urtica dioica</i>	Sisnu	8.05 ± 0.15 ^a	3.87 ± 0.23 ^a	2.92 ± 0.18 ^a
<i>Nasturtium officinale</i>	Simrayo	5.64 ± 0.46 ^b	4.50 ± 0.31 ^b	3.90 ± 0.33 ^b
<i>Diplazium esculentum</i>	Ningro	3.97 ± 0.23 ^c	4.10 ± 0.38 ^{a,b}	2.87 ± 0.13 ^c
<i>Chenopodium album</i>	Bethu	4.80 ± 0.41 ^d	1.77 ± 0.14 ^c	2.10 ± 0.08 ^d

MeOH, methanolic Extract; EtOAC, ethyl acetate Extract; Hexane, hexane extract. Data expressed as mean ± standard deviation; different alphabets (a, b, c, and d) within the same column indicates significantly different at $p < 0.05$.

TABLE 3 | TPC and TFC in selected wild green leafy vegetables of Sikkim Himalayan region.

Green leafy vegetables	TPC (μg GAE/mg of extract)			TFC (μg QE/mg of extract)		
	MeOH	EtOAC	Hexane	MeOH	EtOAC	Hexane
<i>Urtica dioica</i>	45.84 ± 4.04 ^a	22.86 ± 2.50 ^a	15.87 ± 0.96 ^a	11.18 ± 0.42 ^a	14.86 ± 0.66 ^a	10.84 ± 0.44 ^a
<i>Nasturtium officinale</i>	52.06 ± 3.82 ^a	32.76 ± 0.66 ^b	25.40 ± 3.33 ^b	3.32 ± 0.47 ^{b,c}	5.02 ± 0.10 ^b	7.20 ± 0.38 ^b
<i>Diplazium esculentum</i>	31.11 ± 2.86 ^b	16.76 ± 2.75 ^c	11.05 ± 1.90 ^c	2.93 ± 0.08 ^b	3.42 ± 0.15 ^c	1.87 ± 0.22 ^c
<i>Chenopodium album</i>	22.73 ± 1.88 ^c	10.16 ± 0.88 ^d	9.14 ± 0.38 ^c	3.61 ± 0.37 ^c	4.15 ± 0.63 ^c	2.50 ± 0.37 ^d

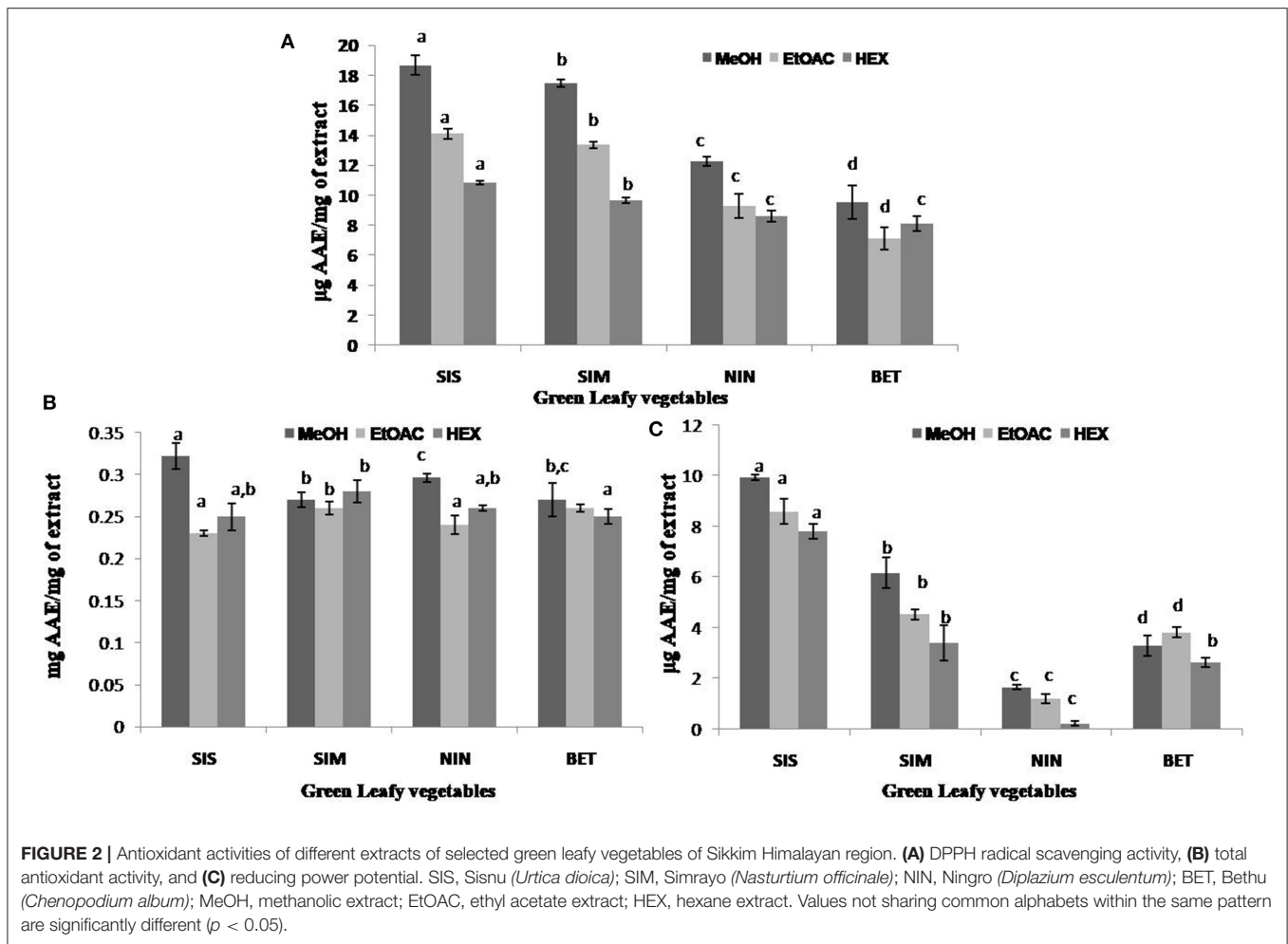
TPC, total phenolic contents; TFC, total flavonoid contents; MeOH, methanolic extract; EtOAC, ethyl acetate extract; Hexane, hexane extract. Data expressed as mean ± standard deviation; GAE, gallic acid equivalent; QE, quercetin equivalent; different alphabets (a, b, c, and d) within the same column indicate significant difference at $p < 0.05$.

compounds in the solvents. In addition, their solubility is contingent on several chemical features including the position of –OH groups, their molecular size, and length of the hydrocarbon chains (40). Polar solvents are often considered suitable to extract most of the phenolic compounds from plant tissues (41, 42). MeOH has been found to be more efficient in extracting high content of low molecular weight phenolics from different plant parts (43). In contrast, flavonoids have an affinity toward both the polar and nonpolar extracting medium because of their diverse chemical structure (O-glycosides and aglycones). Polar solvents have been reported to extract the flavonoid glycosides while nonpolar solvents mostly extract their aglycones (44, 45). Resembling the above-mentioned facts, extracts of GLVs used in the study which were prepared using MeOH and EtOAC (polar extracting medium) appeared to have higher TPC and TFC, respectively. Quite the opposite, TFC in the *N. officinale* hexane extract was found to be higher as compared to other extracts and this can be certainly due to the presence of aglycones in the extract.

The antioxidant activities of the GLV organic extracts were determined using DPPH radical scavenging, TAA, and RPP. Evaluation of antioxidant potential is carried out using different methods as each assay varies in principle and mechanism of action (17). DPPH is a free radical which is a widely applied and acceptable method to study the antioxidant potential of plant extracts (18, 46). The DPPH scavenging activity of the GLV organic extracts ranged from 9.55 to 18.68 μg AAE/mg in MeOH extract, 7.11 to 14.10 μg AAE/mg in EtOAC extract, and 8.09 to 10.85 μg AAE/mg in hexane extracts. Among the test extracts, *U. dioica* MeOH extracts exhibited the highest DPPH radical scavenging activity (18.67 μg AAE/mg of extract), while

the *C. album* EtOAC extract exhibited the lowest activity (7.11 μg AAE/mg of extract) (Figure 2A). TAA of the test extracts ranged from 0.27 to 0.32 mg AAE/mg of extract in MeOH, 0.23 to 0.26 mg AAE/mg of extract in EtOAC, and 0.25 to 0.28 mg AAE/mg of extract in hexane extracts. Among different plants, TAA of the *U. dioica* MeOH extract was found to be higher (0.32 mg AAE/mg of extract), whereas that of the EtOAC extract exhibited the lowest TAA (0.23 mg AAE/mg of extract) (Figure 2B). TAA assay measures the ability of the extract to reduce molybdenum (VI) to molybdenum (V) in acidic condition (47). RPP is an antioxidant method commonly applied for evaluating the presence of reductants, which exhibit antioxidant activity by breaking down of free radical chain on the donation of hydrogen atoms (43). RPP of the test extracts ranged from 1.67 to 9.93 μg AAE/mg of extract in MeOH, 1.2 to 8.60 μg AAE/mg of extract in EtOAC, and 0.23 to 7.80 μg AAE/mg of hexane extracts. Furthermore, the highest RPP was exhibited by *U. dioica* MeOH extract (9.9 μg AAE/mg of extract) and *D. esculentum* hexane extract displayed the lowest RPP (0.23 μg AAE/mg of extract) (Figure 2C). The overall results showed that the organic extracts obtained from *U. dioica* demonstrated relatively significant TPC and TFC, and exhibited promising antioxidant activity. Moreover, the *U. dioica* MeOH extract among others was able to exhibit higher antioxidant activity. Higher antioxidant activity in the *U. dioica* MeOH extract could probably be correlated to higher TPC present in the same (48).

The effects of cooking on the antioxidant potential of *U. dioica* leaf extracts were evaluated using steaming and boiling, two major methods used by the local people. Steaming of *U. dioica* leaves was carried out for 20 min (S20) and 40 min (S40), whereas boiling was carried out for 5 min (B5) and 10 min



(B10) to enable proper cooking. The S40 extract of *U. dioica* demonstrated higher TPC (36.19 μg GAE/mg of extract) and TFC (9.84 μg QE/mg of extract) than S20 having 33.52 μg GAE/mg of extract and 9.04 μg Q/mg of extract, respectively. Conversely, B5 extract showed greater TPC (34.28 μg GAE/mg of extract) and TFC (9.23 μg QE/mg of extract) than B10 which is having TPC and TFC of 34.28 μg GAE/mg and 6.86 μg QE/mg of extracts, respectively. The results demonstrated an increase in TPC and TFC on steam cooking of *U. dioica* leaves (Figure 3A); however, loss of TPC and TFC was observed with leaf extracts prepared using boiled *U. dioica* leaves. The results resemble the findings made by Salamatullah et al. (49), Rocchetti et al. (50), and Gunathilake et al. (51). Similar observations were also witnessed while assessing the effects of cooking on the antioxidant activities (DPPH radical scavenging and TAA) given in Figure 3B. The S40 extract exhibited higher DPPH radical scavenging activity and TAA (18.47 μg AAE/mg of extract and 0.30 mg AAE/mg of extract) than that of the S20 extract (17.28 μg AAE/mg of extract and 0.28 mg AAE/mg of extract). In boiled cooking process, B5 extracts showed higher DPPH (15.54 μg AAE/mg of extract) and TAA (0.3 mg AAE/mg of extract) than B10 (DPPH 13.46 μg AAE/mg of extract and TAA

0.25 mg AAE/mg of extract). Higher boiling time reduced the antioxidant potential of *U. dioica*, as phenolics are released in water used for the boiling method. An increase in antioxidant activity on steam cooking may probably be due to the breakdown of complex structure and release of free form of phenolics on heat treatment (13). Several studies have shown steaming as a useful cooking approach over boiling to retain the antioxidant potential in a few vegetables (14, 49, 51). Therefore, it is essential to standardize the cooking procedure for individual leafy vegetable.

The antioxidant effect of phytochemicals in GLVs will depend not only on their concentration but also on their resistance to the GI environment (13). The sequence of events during cooking followed by GI digestion may lead to an increase or decrease in antioxidant activity (1, 52). Simulated GI digestion of the steam-cooked and boiled leaves was performed using standard referred protocols, and the antioxidant potential (DPPH scavenging and TAA) of the digesta (supernatants and residues) in each case were determined. TPC and TFC were estimated to be higher in steam-cooked and GI digested extracts (0.45 mg GAE/ml extract and 0.13 mg QE/ml extract, respectively) as compared to those estimated in boiled and GI

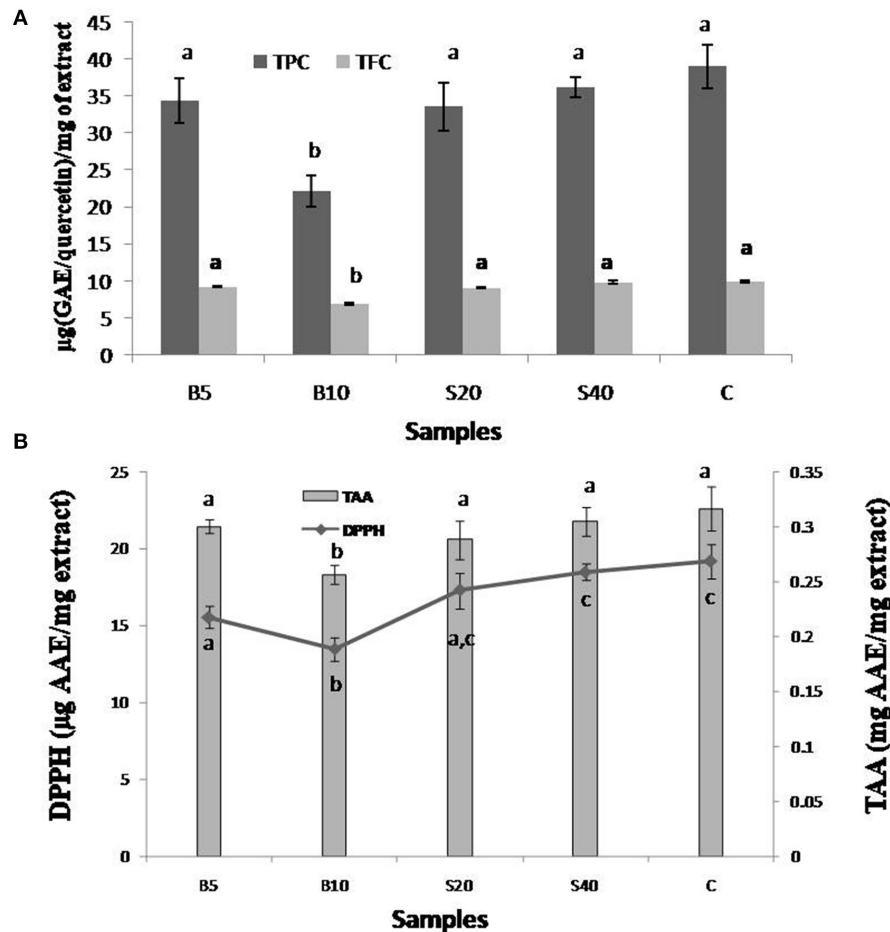
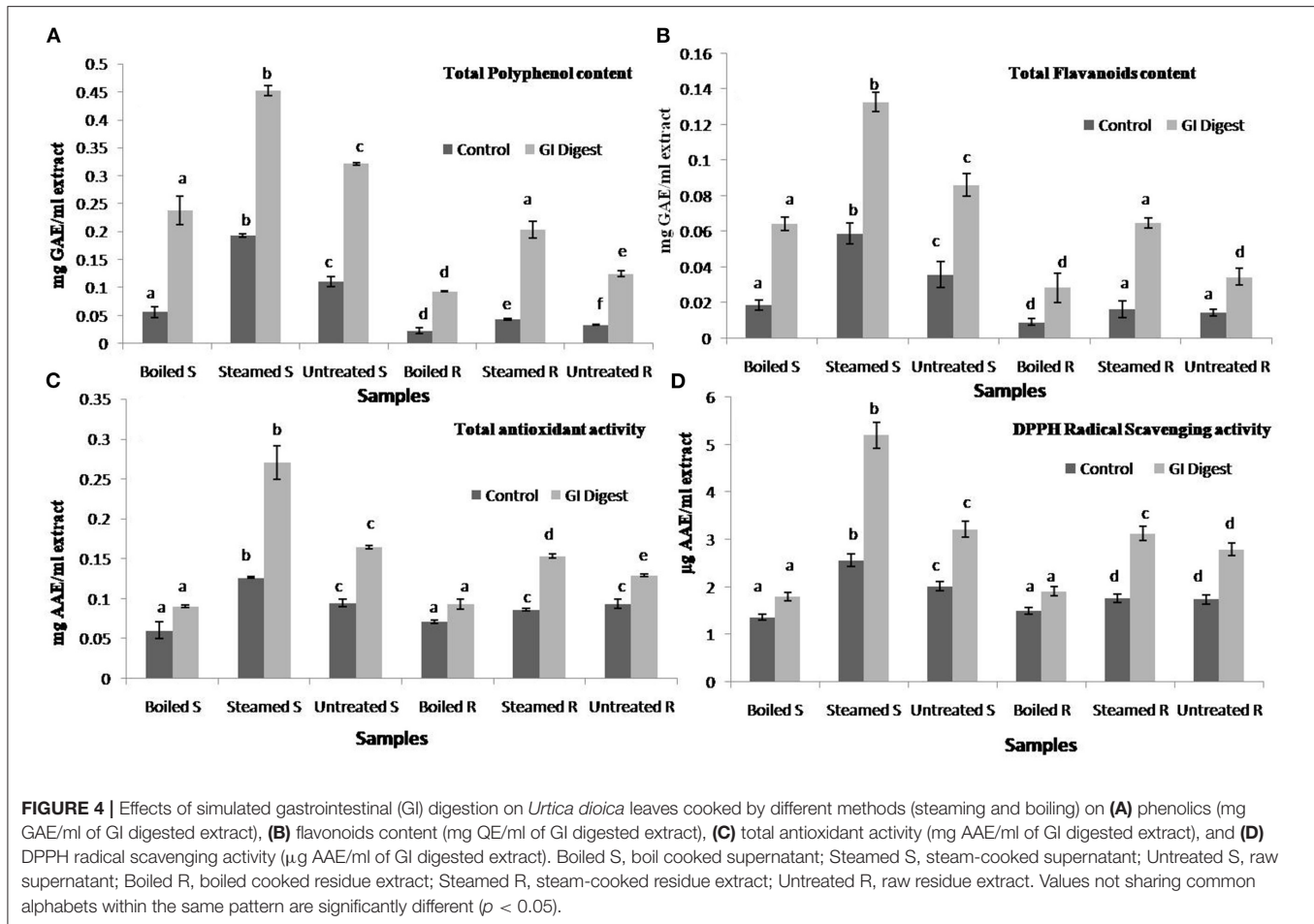


FIGURE 3 | Effects of different cooking methods (steaming and boiling) on (A) phenolic (μg GAE/mg of extract) and flavonoid contents (μg QE/mg of extract) and (B) DPPH scavenging (μg AAE/mg of extract) and total antioxidant activity (mg AAE/mg of extract). TPC, total phenolic contents; TFC, total flavonoid contents; GAE, gallic acid equivalent; QE, quercetin equivalent; AAE, ascorbic acid equivalent. Values not sharing common alphabets within the same pattern are significantly different ($p < 0.05$).

digested (0.24 mg GAE/ml extract and 0.06 mg QE/ml extract, respectively) (Figures 4A,B). Similar findings were also observed in the case of TAA and DPPH scavenging activities where the extracts obtained from the steam-cooked and digested *U. dioica* leaves displayed higher activity (0.27 mg AAE/ml and 5.19 μg AAE/ml of extracts, respectively) as compared to the boiled and GI digested leaves (Figures 4C,D). The overall findings of the study suggest that the leafy vegetables consumed in the SHR could be a great source of natural antioxidant metabolites, namely, phenolics and flavonoids. In addition, steam-cooked *U. dioica* leaves could be preferred for consumption to ensure greater bioavailability of its phenolics and higher antioxidant effects. Furthermore, the findings may encourage the inhabitants of the SHR and other parts of the eastern Himalaya to use particular cooking approaches while cooking *U. dioica* leaves to help retain the phenolics and antioxidant properties.

CONCLUSION

In this study, organic extracts of leafy vegetables commonly used by the local people of the Sikkim Himalayan region were evaluated for their phenolic and flavonoid contents, and antioxidant activity. The MeOH extract of *U. dioica* leaves among others was estimated to have higher TPC, TFC, and displayed significant antioxidant activity. The effects of cooking methods and GI digestion on the TPC, TFC, and antioxidant activity demonstrated that steam-cooked and digested leaves retained greater TPC and TFC, and antioxidant activity. The findings of this study could serve as a source of information in promoting the consumption of leafy vegetables in the SHR region and in the use of a definite cooking process to retain their antioxidant properties. Moreover, further research on the chemical efficacies of steam-cooked *U. dioica* leaves can be evaluated *in vivo*.



DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SS: methodology, investigation, validation, formal analysis, visualization, and writing—original draft. SPad: methodology, investigation, visualization, and data curation. MK: methodology, investigation, and data curation. SPat:

resources, writing—review and editing, and supervision. DS: conceptualization, resources, writing—review and editing, visualization, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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