Emirates Journal of Food and Agriculture. 2022. 34(7): 544-552
doi: 10.9755/ejfa.2022.v34.i7.2897
http://www.ejfa.me/

RESEARCH ARTICLE

In vitro lipoxygenase and hemolysis inhibition by polyphenolic antioxidants from tropical green leafy vegetables

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ABSTRACT

Consumption of green leafy vegetables, a nutrient-dense food, is associated with protective roles in human health and a reduction in risk of cancer, cardiovascular, and other inflammatory diseases. Phytochemical components such as phenolics and flavonoids are said to contribute to these benefits. Data on the anti-inflammatory potential and phenolic content of many Indian green leafy vegetables is still scanty. We investigated eleven tropical green leafy vegetables for their phenolic and flavonoid content along with anti-inflammatory and free radical scavenging properties. Black nightshade had the lowest phenolic and flavonoid content (2.36 mg Gallic Acid Equivalents/g DW and 1.66 mg Rutin Equivalents/g DW), whereas desert horsepurslane (41.73 mg GAE/g DW) and red amaranth (27.18 mg RE/g DW) had the highest phenolic and flavonoid content, respectively. Similarly, the free radical scavenging activity for 2, 2-diphenyl-1-picrylhydrazyl (DPPH) ranged from 10.57 – 84.97 % inhibition and 50.73 – 266.48 µg Trolox Equivalents/g DW for 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate (ABTS) decolorization assay. The phenolic and flavonoid content correlated significantly with the radical scavenging activity. The hemolysis and lipoxygenase inhibition was observed to be lowest in Amaranthus sp. and desert horsepurslane (6.08% and 53.75 %) and highest in dill and fenugreek (69.94% and 78.55 %), respectively. Overall, our study adds to the polyphenolic and antioxidant data available on green leafy vegetables, in addition to highlighting their anti-inflammatory potential.

Keywords: Anti-Inflammation; Free radical scavenging; Green leafy vegetables; Lipoxygenase inhibition; Phenolics

INTRODUCTION

Inflammation is the hallmark of many chronic diseases, including lifestyle disorders. From the simple allergy to the complicated metabolic syndrome, uncontrolled chronic inflammation of the low-grade is observed in the body. The inflammatory response is one of the first defense mechanisms launched by the immune system in injury or trauma. The inflammatory cascade remedies and repairs the injury site to its former pristine level. However, due to various reasons, including stress and nutritional deficiencies, an inflammatory response may refuse to resolve on its own. This situation over a long time leads to chronic inflammation observed in chronic diseases (Furman et al., 2019; Neurath, 2019).

The arachidonic acid pathway is one of the starting points in the initiation of inflammation. Any injury, infection, or trauma releases arachidonic acid from the cell membrane of neutrophils. The arachidonic acid by the cyclooxygenase or lipoxygenase pathway produces prostaglandins and leukotrienes. These are pro-inflammatory molecules that further activate the inflammatory cascade. Hence, blocking or inactivating the activity of these enzymes seems to be a suitable approach for amelioration of inflammation (Garcia-Lafuente et al., 2009). The catalytic site of the soybean lipoxygenase 1 and human lipoxygenases shares a conserved active site iron ligand, a salt bridge, and 3 amino acids. Thus, soybean lipoxygenase resembles human lipoxygenase in its substrate specificity and hence serves as an excellent model to screen anti-inflammatory properties (Prigge et al., 1996).

Natural antioxidants from plants are gaining much interest lately. They are superior to synthetic antioxidants in many ways. They provide nutrition, have consumer acceptance,

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Received: 21 December 2021; **Accepted:** 13 June 2022

and blend well into the food matrix, lending themselves to various food products. Natural antioxidants have since made their way into the food, cosmetic and agricultural industries. Many phytochemicals in plants, namely, phenolics, carotenoids, tocopherols, lignans, and alkaloids, contribute as natural oxygen scavengers (Griffiths et al., 2016). Reactive oxygen and nitrogen species cause the oxidation of lipids, proteins, and DNA. Naturally occurring antioxidants can prevent these highly reactive species from being formed either in the body or in the food preparation by scavenging these wayward species and preventing any damage (Mittal et al., 2014).

Green leafy vegetables are one of the most nutrient-dense foods on the planet. They are a rich source of calcium, iron, magnesium, β-carotene, vitamin C, K, E, dietary fiber, and other trace minerals. Additionally, they provide various bioactive non-nutritive health-enhancing phytochemicals, which are potent antioxidants and have been recognized to prevent lifestyle diseases. The consumption of leafy greens as a part of a balanced diet is recommended by all nutritionists (Jimenez-Aguilar and Grusak, 2017; Kumar et al., 2020). Epidemiologists have found that regular consumption of green leafy vegetables and fruits was responsible for decreased risk of cardiovascular disease, cognitive decline, and many cancers (Mennen et al., 2004; Griffiths et al., 2016; Morris et al., 2018).

Many green leafy vegetables have been in use since folkloric times as a remedy for diseases and disorders. Amaranthus spp. is used against gastroenteritis, gall bladder inflammation, internal bleeding, and arthritis (Achigan-Dako et al., 2014); fenugreek against inflammation, cardiovascular and liver diseases, renal insufficiency, pain, and edema (Kakani and Anwer, 2012); desert horsepurslane against pain, edema, diarrhea, asthma, inflammation, heart and liver diseases, migraine, piles, and rheumatism (Mandal and Bishayee, 2015); black nightshade against diarrhea, ulcers, inflammation, and jaundice (Moyo et al., 2020); dill as an appetite stimulant, against gastrointestinal disorders and rheumatism (Isbilir and Sagiroglu, 2011); Indian borage as a treatment for cough, cold, asthma, bronchitis, epilepsy, skin allergies, and fever (Lukhoba et al., 2006).

Leafy greens like amaranth, fenugreek, and spring onions have gained commercial status and been studied scientifically. However, there are many locally available leafy greens relished seasonally in the southern part of India as part of their regular diet. Most of these plants lack comprehensive data which can substantiate their conventional usage. The present study aimed to check their anti-inflammatory potential and antioxidant profile, considering the possibility of green leafy vegetables as a cheap source of natural antioxidants.

MATERIALS AND METHODS

Linoleic acid, Soybean lipoxygenase, gallic acid, rutin, ascorbic acid, trolox, Folin-Ciocalteau reagent, DPPH (2, 2-diphenyl-1-picrylhydrazyl), and ABTS (2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) were obtained from Sigma, Missouri, USA. Eleven green leafy vegetables were freshly purchased from the local markets of Mysore, India. All other chemicals and solvents were of analytical grade.

Plants extract preparation

Commonly consumed green leafy vegetables (GLVs) like *Anthenum graveolens* L. (Dill), *Amaranthus blitum* L. (Green amaranth), *Amaranthus dubius* Mart. Ex Thell. (Chinese spinach), *Amaranthus blitum* var. *oleracea* L. (Red amaranth), *Trigonella foenum-graecum* L. (Fenugreek), *Solanum americanum* Mill. (Black nightshade), *Trianthema portulacastrum* L. (Desert horsepurslane), *Allium cepa* L. (Onion leaves), *Sesbania grandiflora* (L.) Pers. (Vegetable hummingbird), *Basella alba* L. (Malabar spinach), *and Plectranthus amboinicus* (Lour.) Spreng. (Indian borage) were purchased from the local markets of Mysuru, India (Table 1). The GLVs were identified and stored in the herbarium of The Department of Studies in Botany, University of Mysuru, India. They were washed, dried at 55 ± 2 °C in a hot air drier, and powdered and

Table 1: The Green leafy vegetables (GLVs) selected in the current study and their details

No.	Symbol	English name	Scientific name	Family
1	GLV1	Dill	*Anethum graveolens* L.	Apiaceae
2	GLV2	Green amaranth	*Amaranthus blitum* L.	Amaranthaceae
3	GLV3	Fenugreek	*Trigonella foenum-graecum* L.	Fabaceae
4	GLV4	Black nightshade	*Solanum americanum Mill.*	Solanaceae
5	GLV5	Chinese spinach	*Amaranthus dubius* Mart. ex Thell.	Amaranthaceae
6	GLV6	Red amaranth	*Amaranthus blitum* var. *oleracea* L.	Amaranthaceae
7	GLV7	Spring onions	*Allium cepa* L.	Amaryllidaceae
8	GLV8	Vegetable hummingbird	*Sesbania grandiflora* (L.) Pers.	Fabaceae
9	GLV9	Desert horsepurslane	*Trianthema portulacastrum* L.	Aizoaceae
10	GLV10	Malabar spinach	*Basella alba* L.	Basellaceae
11	GLV11	Indian borage	*Plectranthus amboinicus* (Lour.) Spreng.	Lamiaceae

Table 2: The correlation coefficient of the antioxidant constituents and the free radical scavenging capacities of the GLVs

	TPC (mg/g DW)	TFC (mg/g DW)	DPPH (%)	TE (μg/g DW)
TPC (mg/g DW)	1			
TFC (mg/g DW)	0.80*	1		
DPPH (%)	0.65*	0.56*	1	
TE (μg/g DW)	0.71*	0.79*	0.58*	1

TPC (mg/g DW) - Total phenolics, TFC (mg/g DW) - total flavonoids, DPPH (%) – Percentage scavenging of DPPH, TE (μg/g DW) – Trolox equivalents of ABTS scavenging capacity, * - Significant at $P<0.05$

stored at -20 °C. All the powders were extracted in distilled water and 70% ethanol. Briefly, 10 g of each powder was dissolved in either 100 mL water or 70% ethanol and kept on a shaker for 8 h at 15 ± 2 °C. The extracts were filtered using a muslin cloth, and the residue was re-extracted twice in a similar manner with the respective solvents. The filtrates were pooled and filtered with Whatman's No. 1 filter paper. The solvents were evaporated *in vacuo* using rota-vapor, and the resulting residues dissolved in 1% DMSO. The *in vitro* assays were carried out with these extracts.

Eleven leafy greens extracted in two different solvents resulted in 22 extracts, which were screened for *in vitro* anti-inflammatory activity and antioxidant properties.

RBC membrane stabilization assay

RBC preparation

The erythrocytes suspension was prepared by a procedure described by Gunathilake et al. (2018). The Institutional Ethics Committee approved all experimental protocols (Registration No. JSSMC/IEC/2509/Aca Study 04/2018-2019, JSS Medical College, Mysuru). Blood was collected in tubes having anti-coagulant from healthy human volunteers. It was mixed with an equal volume of sterile Alsever's solution and centrifuged at 3000 rpm for 10 min. The packed red blood cells (RBCs) were washed with 0.9% saline three times, and 10% v/v RBC suspension was made with saline. This human RBC suspension was used for the hemolysis assays.

Hypotonic hemolysis assay

The hypotonic hemolysis assay was carried out as described by Rajarathinam and Dronamraju (2018), with some modifications. The reaction mixture consisted of 1 mL phosphate buffer (0.15 M, pH 7.4), 2 mL of 0.36% NaCl, 1 mL of 500 μg/mL extract and 0.5 mL human RBC (10% v/v in saline). The extracts were tested against standard anti-inflammatory NSAID Sodium diclofenac (50 and 100 μg/mL). Instead of the extract, 1 mL of distilled water served as positive hemolysis control (100% lysis). The control tube contained phosphate buffer, 0.36% NaCl, and RBC. The reaction mixture was incubated at 37 °C for 30 min followed by centrifugation at 3000 rpm for 5 min. Absorbance was read at 560 nm. The inhibition of hemolysis under hypotonic solution was calculated according to the formula,

Inhibition of Hemolysis = (Abs. of control-Abs. of test)/ Abs. of control* 100

Heat-hemolysis assay

The heat-induced hemolysis assay was checked by the procedure described by Gunathilake et al. (2018), with some modifications. The reaction mixture consisted of 1 mL phosphate buffer (0.15 M, pH 7.4), 1 mL of 500 μg/mL extract and 0.5 mL human RBC. As a control, instead of extract, normal saline was used. Sodium diclofenac (50 and 100 μg/mL) was the standard drug. The tubes were incubated at 54 °C for 30 min, followed by centrifugation at 3000 rpm for 5 min. Absorbance was taken at 560 nm, and the percentage of heat-hemolysis inhibition was calculated according to the formula given above.

Lipoxygenase inhibition assay

Lipoxygenase inhibition assay was used as a screening tool for checking the anti-inflammatory activity in the various leafy greens extracts. Lipoxygenase (LOX) activity was assayed, according to Axelrod et al. (1981). For its inhibition studies, the enzyme was incubated with the different extracts (1 mg/mL) for 3 min in 0.2 M borate buffer (pH 9.0), and the addition of linoleic acid started the reaction. A differential decrease in absorbance was monitored between the control (without the extracts) and the test extracts at 234 nm. The reaction was followed for 3 min at 25 ºC.

Total Phenolic Content (TPC)

The total phenolic content of the aqueous and ethanolic extracts was determined by the Folin–Ciocalteu method (Herald et al., 2012). The TPC was estimated by a microplate method. Gallic acid (3.125 – 200 μg/mL) was used as the standard. Double distilled water (75 μL) was added to all the wells, followed by 25 μL of standard/sample (1 mg/mL) and 25 μL of 1:1 diluted Folin-Ciocalteu reagent. Appropriate solvent blanks were also set up. The plate was incubated at room temperature (~ 27 ± 2 °C) for 6 min, followed by the addition of 100 μL of 10% sodium carbonate solution. The plate was gently tapped on the sides to mix the contents and incubated at room temperature for 90 min in the dark. The absorbance was read at 765 nm in a microplate reader (TECAN, Mannedorf, Switzerland) after carefully shaking the plate for 30-60s. The data were stated as microgram Gallic Acid Equivalents (GAE)/g dried weight.

Total flavonoid content (TFC)

The total flavonoid content of the aqueous and ethanolic extracts was determined according to the aluminum

chloride colorimetric method described by Herald et al. (2012). The TFC was estimated by a microplate method. Rutin (3.125 – 200 µg/mL) was used as a standard. Double distilled water (100 µL) was added to all the wells, followed by 25 µL of standard/sample (1 mg/mL) and 10 µL of 5% sodium nitrite solution. Appropriate solvent blanks were also set up. The plate was incubated at room temperature (~ 27 ± 2°C) for 5 min followed by 15 µL of 10% Aluminium chloride solution. The plate was tapped gently to mix the contents and further incubated for 6 min. After incubation, 50 µL each of 1 M sodium hydroxide and distilled water was added to each well, followed by an absorbance reading at 510 nm against the reagent blank. The data were expressed as milligram Rutin Equivalents (RE)/g dry weight.

Free radical scavenging activity by DPPH● (2, 2-diphenyl-1-picrylhydrazyl) radical

In this assay, antioxidant or reducing compounds reduce the purple chromogen radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) to a consequent pale yellow hydrazine. The procedure described by Herald et al. (2012) was used with some modifications. A DPPH· solution of 60 µM in methanol was made, and 200 µL of this solution was added to all the wells except the blank wells in a microtitre plate. Sample, control, and standard solutions (50 µL) were added to the respective wells and kept in the dark at room temperature for 20 min. A decrease in absorbance at 517 nm of DPPH was observed towards the completion of the incubation period. Vitamin C (35.5-568 µM) was the standard, and aqueous and 70% alcoholic extracts of all the leafy greens (1 mg/mL) were analysed. The activity was calculated as effective concentration EC50 of the sample by the following formula:

$$\% \text{ Effective concentration} = [1-(A_{Sample} - A_{Blank})/(A_{Control} - A_{Blank})] *100$$

$ABTS^+$ (2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)) radical cation or Trolox equivalent antioxidant capacity (TEAC) assay

The assay is based on the interaction between an antioxidant and ABTS radical cation ($ABTS^+$) with absorption maxima at 734 nm. The $ABTS^+$ is produced by reacting a 7.4 mM ABTS stock solution with a 2.6 mM potassium persulfate stock solution in a 1:1 proportion for 12-16 h in the dark. This solution is then diluted by mixing 1 mL $ABTS^+$ solution with an appropriate volume of methanol to get ~0.700 OD reading in UV spectrometer. The assay described by Thaipong et al. (2006) with modifications for microplate was utilized. The $ABTS^+$ solution (200 µL) was added to all the wells except for the blank wells, which contained only vehicle solvent and methanol. Sample, control, and standard (50 µL), in quintuplets, were added to the respective wells, and a decrease in absorbance was noted at exactly 10 min at 734 nm. Many natural antioxidants do not attain equilibrium in 10 min of reaction time. Hence, the final reading was taken after 2 h of incubation in the dark. The conversion of the blue-green $ABTS^+$ radical form to its neutral colorless form conveys the antioxidant property of the sample. Trolox (25-200 µM) was used as standard. Both the extracts of the 11 GLVs were tested for ABTS radical scavenging property. The results were expressed as mg Trolox Equivalents (TE)/g of DW.

Statistical Analysis

All the experiments were conducted in triplicates, and the data was reported as mean ± standard deviation. One-way analysis of variance (ANOVA) was employed to analyze the statistical differences amongst the 2 extracts for all the experiments. Results were considered significant when $P < 0.05$. The correlations between the phenolic contents and antioxidant attributes were determined by Pearson's correlation coefficient.

RESULTS AND DISCUSSION

Green leafy vegetables are commonly consumed as part of a regular diet. They provide maximum nutrition per calorie than any other food. They contain significant amounts of vitamin A, C, E, and K and many B vitamins. Leafy greens are a rich source of minerals, including calcium, iron, magnesium, and potassium. They are high in fiber while being extremely low in carbohydrates and fat (Jimenez-Aguilar and Grusak, 2017; Kumar et al., 2020).

Leafy greens commonly eaten in the Indian sub-continent like dill, fenugreek, amaranths, spring onions, and Malabar spinach were checked for their anti-inflammatory property in the current study. Along with these popular greens, some locally consumed greens like black nightshade, desert horsepurslane, Indian borage, and vegetable hummingbird were also examined.

Membrane stabilization assay

Membrane stabilization assay is used as an *in vitro* anti-inflammatory assay because the RBC membrane is structurally similar to the lysosomal membrane. The integrity of the lysosomal membrane is of utmost importance during inflammation, wherein the lysosomes of activated neutrophils rupture to release degradative enzymes and chemicals, thereby causing tissue damage. Thus, the lysosomal membrane stabilization limits the harmful effects of an inflammatory response (Gunathilake et al., 2018). Compounds that stabilize the RBC membrane also interfere with the release of phospholipases that initiate the formation of inflammatory mediators. Membrane

stabilization of RBC under hypotonic and heat-induced conditions relates to the principle that RBC membrane ruptures due to fluid accumulation inside the cell and heat denaturation, respectively.

Both the extracts of leafy greens stabilized the human RBC membrane under hypotonic hemolysis conditions. Specifically, aqueous extracts of dill, green amaranth, vegetable hummingbird, and desert horsepurslane stabilized more than 50% of RBC in the hypotonic stabilization assay (Fig. 1A). Similarly, 70% alcoholic extract of dill, fenugreek, red amaranth, desert horsepurslane, and Indian borage stabilized more than 50% of RBC compared to standard sodium diclofenac (100 µg/mL) with 63.51% of RBC stabilization under hypotonic conditions.

Only dill, green amaranth, Chinese spinach, fenugreek, spring onion, and Indian borage stabilized the RBC membrane compared to standard sodium diclofenac (100 µg/mL) which provided 89% protection in heat-induced hemolysis assay (Fig.1B). Heat is a harsh method of hemolysis, which could be why most GLVs (500 µg/mL) could not prevent RBC hemolysis under heating conditions. Most GLVs provided RBC stabilization at 1 mg/mL against heat hemolysis (Data not shown). Gunathilake et al. (2018) showed that vegetable hummingbird provided only 10% stabilization of RBC against heat-induced hemolysis at 3 mg/mL whereas, in our experiment, it was ineffective at 500 µg/mL. Stabilization of RBC membrane indicates the anti-inflammatory nature of the green leafy vegetable extracts.

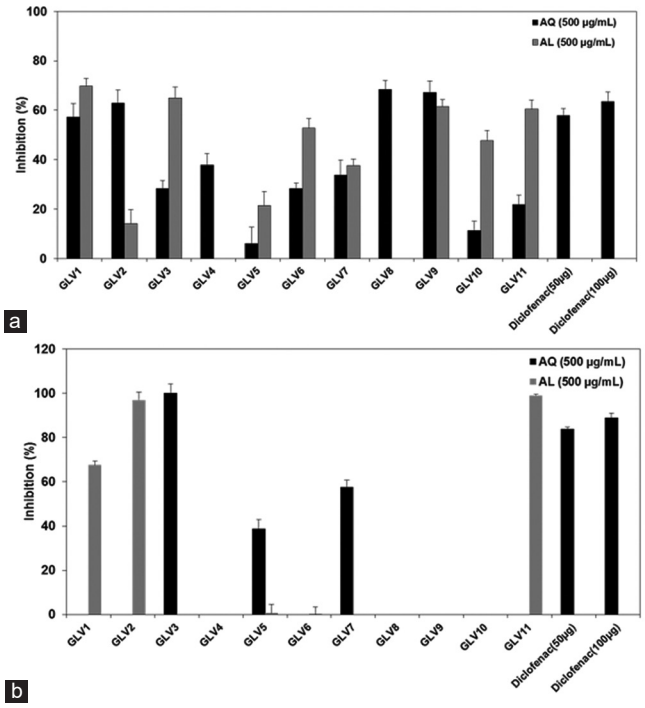

Fig. 1. RBC membrane stabilization ability of the GLVs and standard diclofenac. Inhibition of hypotonic hemolysis (a) and heat-induced hemolysis (b) by the GLVs. Data are presented as mean ± standard deviations.

Lipoxygenase assay

Lipoxygenase is one of the enzymes triggered in the early stages of inflammation. In the event of sudden trauma or injury, Phospholipase A_2 degrades membrane lipids into fatty acids, including linoleic acid and arachidonic acid. Lipoxygenase converts the fatty acids into different leukotrienes. Leukotrienes are the early pro-inflammatory molecules that orchestrate the body's inflammatory response, resolving the injury or trauma (Garcia-Lafuente et al., 2009). However, uncontrolled inflammation, such as seen in chronic inflammation, is harmful, and there arises the need for natural lipoxygenase inhibitors.

GLVs tested for their lipoxygenase inhibition showed moderate to high inhibition, at par with the standard drug, sodium diclofenac (Fig. 2). Lipoxygenase inhibition ranged from 53.75–72.11% and 64.05–78.55% in the aqueous and ethanolic extracts, respectively. For most samples, the ethanolic extract showed significantly better lipoxygenase inhibition than the aqueous extract, except for black nightshade and Malabar spinach, which had similar inhibition for both the extracts. Aqueous ethanolic solutions are well-known to extract enhanced bio-actives than purely aqueous or ethanol solutions. This could be the reason that 70% ethanolic extracts of GLVs resulted in better lipoxygenase inhibition than the aqueous extracts.

There is not much information about the Soybean lipoxygenase (LOX1) inhibition by Indian GLVs. Akula and Odhav (2008) tested methanolic extracts of several African GLVs against soybean LOX inhibition. Gunathilake et al. (2018) found that 70% methanolic extract of vegetable hummingbird exhibited 30% LOX1 inhibition at 3 mg/

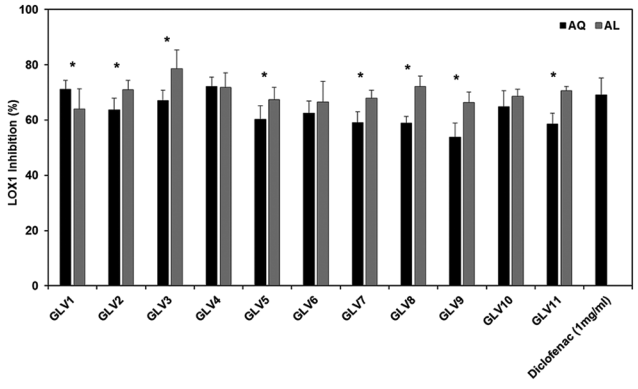

Fig. 2. Lipoxygenase inhibition ability of GLVs and standard diclofenac. Data are presented as mean ± standard deviations. * - Inhibition by LOX-1 by aqueous and 70% alcoholic extracts are significantly different, P<0.05

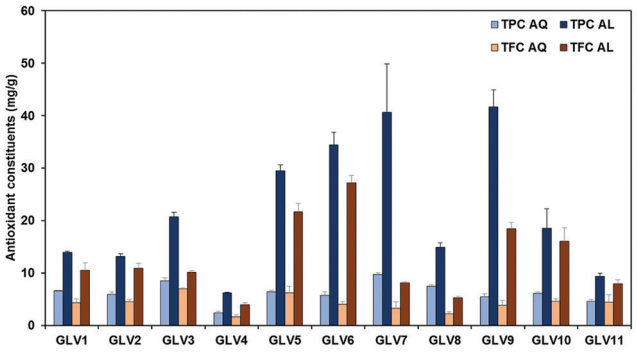

Fig. 3. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of Aqueous (AQ) and alcoholic (AL) extracts of GLVs. Data are presented as mean ± standard deviations.

mL concentration. Loncaric et al. (2021) reviewed several GLVs having LOX1 inhibitory property and found that in the Fabaceae family, methanol extract of the *Cassia alata* leaves showed LOX inhibition of 67% at 100 µg/ml; methanol extract of *Pterocarpus erinaceus* roots exhibited 45% LOX inhibition at 100 µg/ml whereas ethanol extract of *Crotalaria longipes* had the highest inhibition of 72% at 500 µg/ml. Amaranthus spp. has variously been reported to have LOX1 inhibition (Akula and Odhav, 2008; Salvamani et al., 2016; Majdoub et al., 2017).

The lipoxygenase enzyme is implicated in the onset of several inflammatory diseases, including allergies, asthma, pain, edema, and arthritis (Furman et al., 2019; Neurath, 2019). The inhibition of LOX by Amaranthus spp. (60 – 70%), fenugreek (66 – 78%), Indian borage (58 – 70%), and black nightshade (72%) substantiate their traditional usage against such inflammatory diseases and disorders.

Total Phenolics and flavonoid content (TPC & TFC)

Green leafy vegetables are known to be rich in phenolics and antioxidant properties. The phenolic and flavonoid content in the GLV extracts used for the anti-inflammatory property was determined. The total phenolic content ranged from 2.36–12.97 mg GAE/g DW and 6.17–41.73 mg GAE/g DW in aqueous and 70% ethanol extracts, respectively (Fig. 3). Phenolic content in African GLVs ranged from 12.5–26.2 mg/g DW and 24.1–40.6 mg/g DW in aqueous and methanol extracts, respectively (Akula and Odhav, 2008). Comparable phenolics were stated by Mohan Kumar et al. (2018) in Indian GLVs ranging from 4.4–10.2 mg/g DW.

Spring onions had the highest phenolics, 9.75 mg GAE/g DW in water extract, whereas desert horsepurslane had the highest phenolics, 41.73 mg GAE/g DW in 70% ethanolic extract. Black nightshade had the lowest phenolic level of 2.36 and 6.17 mg GAE/g DW in both aqueous and ethanolic extracts. Our finding of TPC of spring onion 40.53 mg/g DW is directly in line with the findings of Issa et al. (2013), ranging from 16.65–22.88 mg/g DW for different varieties of spring onions. Water extracts of Amaranthus spp. and vegetable hummingbird were comparable, whereas the 70% ethanolic extracts in our study yielded more phenolics (Mohan Kumar et al., 2018). Comparative values of the phenolic content of 6.22 mg GAE/g DW in Malabar spinach were estimated by Yadav et al. (2013) against our observation of 6.11 mg/g DW. Dill leaf was found to have 6.58 and 13.91 mg GAE/g DW of total phenols in water and 70% ethanol extracts, respectively. Contrastingly, there have been reports of dill leaves having a varied phenolic profile from 0.94 mg GAE/g DW (Queralt et al., 2015) to 71 mg GAE/g DW (Isbilir and Sagiroglu, 2011).

Amaranthus species were observed to have a higher phenolic content, especially in the 70% ethanolic extract ranging from 13.13–34.49 mg GAE/g DW. Analogous results of total phenolics ranging from 11.8 – 69 mg/g DW were reported in Amaranthus spp. (Jimenez-Aguilar and Grusak, 2017; Yadav et al., 2013; Karamac et al., 2019). In contrast, Sarker and Oba (2019, 2020) observed low phenolics in Amaranthus spp. from 0.5 – 2.5 mg GAE/g DW in the 90% methanol extract. Gupta and Prakash (2008) reported the phenolic content of 4 GLVs, out of which fenugreek values were comparable to our findings of 8.47 mg GAE/g DW vs. 6.37 mg Tannic acid/g. However, it differed remarkably for Amaranthus spp, wherein we observed a range of 5.78–34.49 mg GAE/g DW phenolic content against reported 11.0 mg tannic acid/g. Additionally, it is not clear in their study whether the data presented is on a dry weight basis or wet weight basis.

The total flavonoids in the GLVs ranged from 1.66–6.95 mg RE/g DW and 3.91–27.18 mg RE/g DW in aqueous and 70% ethanolic extracts, respectively (Fig. 3). Black nightshade had the lowest flavonoid content 1.66 and 3.91 mg RE/g DW in both the extracts, whereas fenugreek and Amaranthus spp. had the maximum flavonoid content (6.95 & 10.08 and 4.02–27.18 mg/g DW) in aqueous and alcoholic extracts, respectively. Saikia and Deka (2015) also found similar ranges of flavonoids from 0.65 to 7.72 mg QE/g DW in the GLVs of North-East India. Padmashree et al. (2014) also found similar total flavonoid content of 2.73 mg/g in black nightshade. Jimenez-Aguilar and Grusak (2017) computed the flavonoid content of various Amaranthus species to be 10-40 mg CE/g DW which is close to what we observed from 10.84 – 32.74 mg RE/g DW. Similarly, the flavonoid content in spring onions was found to be 3.80–5.69 mg/g DW, which is close to what we observed (Issa et al., 2013).

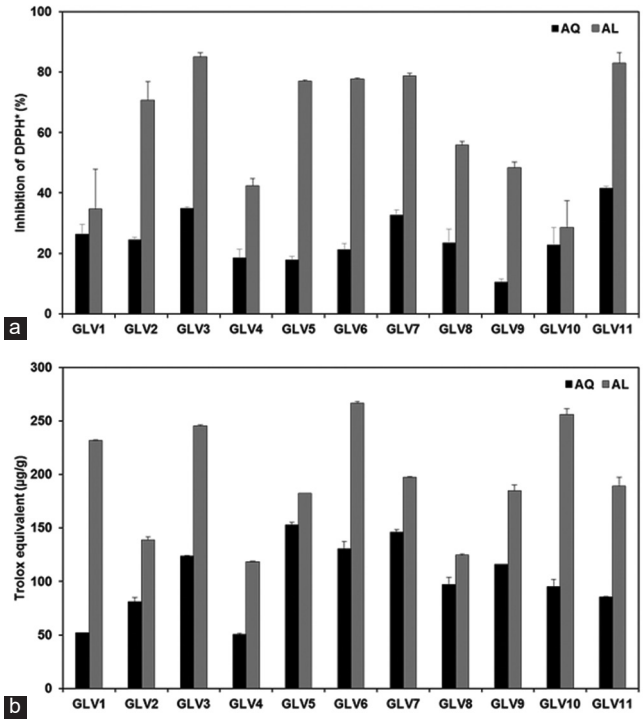

Fig. 4. Antioxidant activity of GLVs. DPPH (a) and ABTS (b) radical scavenging activity of aqueous (AQ) and alcoholic (AL) extracts of GLVs. DPPH scavenging activity is presented as percentage inhibition with ± standard deviations, and ABTS radical scavenging activity is presented as Trolox equivalent (mg/g) ± standard deviations.

Phenolics and flavonoids were extracted efficiently ($P<0.001$) by 70% ethanol than by water in 9 GLVs out of the eleven. Similar changes in efficiencies were reported by Akula and Odhav (2008) and Jaiswal et al. (2017) in water and ethanol extraction. There are considerable differences in the total phenolic content being reported across the literature. This occurs due to many variables present in the studies - the solvent used for extraction, extraction method, extraction time and the standards used for estimation, and the estimation methods themselves. Another important factor contributing to the variations in the values may be due to the reason that phenolic molecules may be water-soluble, lipid-soluble, insoluble, or bound to cell matrices. Hence, the efficiency of extraction also contributes to the quantitative analysis of the phenolic compounds along with the natural variation found among the sources (Gupta and Prakash, 2009; Mohan Kumar et al., 2016).

Antioxidant assays

High levels of reactive oxygen species can react and oxidize cell DNA, proteins, and lipids, resulting in DNA breakage, cell aging, and oxidative stress-related inflammatory diseases (Mittal et al., 2014). Important dietary antioxidants such as ascorbic acid, polyphenols, α-tocopherol, and carotenoids can scavenge free radicals and prevent, inhibit or delay oxidation (Sharifi-Rad et al., 2020). Phenolics, in particular, have excellent redox properties as hydrogen donors, singlet oxygen quenchers, heavy metal chelators, reducing agents, and hydroxyl radical quenchers (Gupta and Prakash, 2009).

The antioxidant activity of the GLVs in our study was studied by DPPH and ABTS radical scavenging assays. The DPPH radical scavenging activity ranged from 10.57–41.62 % and 28.63–84.97 % in the aqueous and ethanolic extracts, respectively (Fig. 4A). Ethanolic extracts showed significantly higher DPPH radical scavenging properties than water extracts. A similar pattern of variation was observed in African GLVs (Akula and Odhav, 2008) and Indian GLVs (Jaiswal et al., 2017).

High DPPH scavenging potential was observed in Amaranthus spp. (70.75–77.72%), fenugreek (84.97%), spring onions (78.77%), and Indian borage (83.04%) in the ethanolic extracts. Lower ranges were observed in desert horsepurslane (10.57%), Malabar spinach (22.82%), black nightshade (18.56%), and dill (26.40 %) in the aqueous extract. The antioxidant properties of fenugreek and Amaranthus spp. matched with the results given by Jaiswal et al. (2017) and Dasgupta and De (2007). However, the drastically lower antioxidant activity of 23.89 % (fenugreek) and 41.25% (Amaranthus sp.) was reported by Gupta and Prakash (2008). The antioxidant property of spring onions at 68.8–83.1% was similar to that reported in our study (Issa et al., 2013). Siddhuraju et al. (2014) observed the DPPH activity to be 30% in ethanolic extract of the vegetable hummingbird, which is lower than the 55.93% which we observed. In our study, dill leaves had a DPPH scavenging potential of 26.40 and 34.75 %, which is close to the observations made by Queralt et al. (2015) but drastically less than 79.73 % and 87.22 % in water and ethanol extracts reported by Isbilir and Sagiroglu (2011).

The antioxidant scavenging activity using ABTS radical ranged from 50.73 – 152.49 TE µg/g DW and 118.30–320.97 TE µg/g DW in the aqueous and ethanolic extracts, respectively (Fig. 4B). Amaranthus spp. (129.06–266.48 TE µg/g DW) had higher TEAC than other GLVs. Similar levels were found in *Amaranthus hybridus* and *Amaranthus caudatus* (Adefegha and Oboh, 2011; Karmac et al., 2019). However, Sarker and Oba (2019 and 2020) observed lesser antioxidant values (26.96-68.89 TE µg/g DW) in the Amaranthus spp., which they studied. Dill leaves had a 231.93 TE µg/g DW in alcoholic extract, similar to the reported value (Queralt et al., 2015). The ABTS radical scavenging of the ethanolic extract in our study is comparable with the reported values (Jaiswal et al., 2017); however, they observed more antioxidant capacity in the water extract than in the

ethanolic extract for fenugreek and Amaranthus. This is contrary to our findings. We significantly observed more antioxidant capacity in the ethanolic extracts than the water extracts. In fact, the percentage scavenging activity of many of the ethanolic extracts of the GLVs, namely dill, fenugreek, Amaranthus spp., and spring onions, was nearly 100%. Indian borage exhibited high free radical scavenging activity in both DPPH and ABTS assays (83.04% of DPPH inhibition and 189.15 TE μg/g DW in ABTS assay).

The correlation of antioxidant constituents and the activity for all the GLVs are given in (Table 2). A significant positive correlation ($P < 0.05$) was observed between the total phenolic content and total flavonoid content and the antioxidant assays, DPPH and ABTS. A significant correlation ($P < 0.05$) was also observed between the antioxidant assays DPPH and ABTS. The correlations correspond with the results of many other researchers of GLVs (Sreeramulu et al., 2013; Sarker and Oba, 2020).

CONCLUSION

The current study demonstrated that the unexplored leafy greens such as desert horsepurslane, vegetable hummingbird, and Indian borage had equivalent or higher phenolics and antioxidant capacity than the commercial greens Amaranthus, fenugreek, and spring onions. The antioxidant activity also correlated significantly with the phenolic and flavonoid contents. Our work confirmed that hydroalcoholic solvent was significantly better at extracting polyphenols from green leafy vegetables than aqueous solvent. The preliminary in vitro assays of anti-inflammation validate the traditional usage of these leafy greens to ameliorate inflammatory diseases like pain, edema, arthritis, or asthma. The lipoxygenase inhibition exhibited by the leafy greens points out their anti-inflammatory potential. Further anti-inflammatory studies in *in vivo* model would help to validate the observed effect. The study provides new insights into the disease-fighting potential of these leafy greens along with their antioxidant capacity. This information will be helpful to nutritional and pharmaceutical researchers for improving various nutraceutical products.

ACKNOWLEDGEMENT

The authors are grateful to Director, CSIR-CFTRI, Mysuru, for providing the infrastructure to carry out the research. The authors express their sincere thanks to Prof. Suresha R. N., Department of Pharmacology, JSS Medical College, Mysuru, for assisting in hemolysis assay. The first author is thankful to the Council for Scientific and Industrial Research, India, for the fellowship award.

Conflicts of interest

The authors declare they do not have any conflict of interest.

Authors' contributions

SNM conceptualized the work. Most of the data were acquired by KBS and prepared the original draft. Reviewing and editing, supervision, data curation, and making the final draft were by SME. All authors have read and agreed to the submitted version of the manuscript.

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