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Sukriti Das
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Ipsita De
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Gargi Nag
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Swagata Karak
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Moumita Biswas
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Susmita Das
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Bratati De
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Corresponding Author:
Bratati De
Department of Botany, University of Calcutta. 35 Ballygunge Circular Road, Kolkata. West Bengal, India

Phenolic profile and inhibitory activity of leafy vegetables against enzymes related to stress induced diseases: A comparative study

Sukriti Das, Ipsita De, Gargi Nag, Swagata Karak, Moumita Biswas, Susmita Das and Bratati De

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Abstract
Leafy vegetables have immense medicinal properties apart from their nutrient contents. So, extracts of fourteen leafy vegetables of India were studied for their properties to inhibit enzymes that are involved in different stress induced diseases such as diabetes, obesity and associated diseases, neurodegenerative diseases, hepatic disorders. The extracts were examined for their inhibition properties against α-glucosidase, α-amylase, lipase, acetylcholinesterase and β- glucuronidase. Most of the extracts inhibited the enzymes in concentration dependent manner, except a few which showed less or no activity. Among all the tested species, only *Bauhinia acuminata* exhibited α-amylase inhibition with a high IC50 value of 465.57±8.81 µg/ml. *Bacopa monnieri* had highest α-glucosidase inhibition property. Lipase inhibitory activities of the extracts of *Ipomoea aquatica*, *Nyctanthes arbortristis*, *Moringa oleifera*, *Centella asiatica* and *Hygrophila schulli* were found to be significantly higher than that of orlistat. Methanolic extracts of *Nyctanthes arbortristis*, followed by *Ipomoea aquatica*, *Bauhinia accuminata*, *Hygrophila schuli*, and *Trigonella foenum-graecum* showed promising activity against acetylcholinesterase. However, the activities were lower than that of the well-known acetylcholinesterase inhibitor galanthamine. *Coriandrum sativum*, *Enhydra fluctuans*, *Hygrophila schulli*, *Ipomoea aquatica*, *Nyctanthes arbortristis*, *Paederia foetida*, and *Trigonella foenum-graecum* were found to have higher β-glucuronidase inhibition properties than silymarin, a plant sourced β-glucuronidase inhibitor. Gas chromatography - mass spectrometry indicated the presence of different phenolic constituents having enzyme inhibition properties in the plants.

Keywords: Leafy vegetables, enzyme inhibition, phenols, bioactivity

Introduction
The qualitative value of plants, other than some nutrients, is due to the presence of their metabolites [1]. In addition to nutrients, the non-nutrient phytochemicals or plant specialized metabolites present in plants are shown to have immense bioactivities [2]. In addition to the antioxidant properties of edible plants, inhibitors of enzymes are of potential value to control and treat several diseases such as diabetes, obesity, as well as neurological, hepatic and cardiac disorders.

Diabetes is indicated by increased blood glucose levels (Hyperglycemia). It is a metabolic disorder, caused by defect in release of insulin, resulting in changes in metabolism of carbohydrates, proteins, and fats [3]. One of the available therapies is to inhibit dietary starch degradation by inhibiting the carbohydrate digesting enzymes α-glucosidase and α-amylase by acarbose, and voglibose etc. But these inhibitors have side effects [4]. Carbohydrates that are obstructed from absorption, are fermented by colonic bacteria resulting in discomfort [5]. The major part of human diet consists of carbohydrates, especially polysaccharides. For intestinal absorption, carbohydrates are to be hydrolyzed to monosaccharides by α-amylase and α-glucosidase. Ultimately released glucose units are absorbed by the gut resulting in postprandial increase of blood sugar level. Inhibition of these two enzymes slows down carbohydrate digestion and absorption of sugars [6, 7]. Acarbose is the inhibitor of α-glucosidase in the intestine and efficiently suppresses postprandial hyperglycemia in type 2 diabetes [8] by lowering rate of glucose absorption.

Obesity is associated with the comorbid conditions such as cardiac disorders, muscular disorders, diabetes, obstructive sleep apnea, respiratory problems, and some cancers [9]. Increased intake of lipids, although an important component of human nutrition, increases the

level of blood cholesterol and triglycerides causing hyperlipidemia and obesity [10-12]. Inhibition of pancreatic lipase inhibits absorption of triglycerides [13]. Orlistat, a drug often used for the treatment of obesity, inhibits lipase [14, 15]. However, this medication is associated with gastrointestinal side effects [16].

Acetylcholinesterase (AChE) is the enzyme that hydrolyzes the neurotransmitter acetylcholine. The neuropsychological impairment in Alzheimer's Disease (AD) are the consequences of cholinergic disturbances. One treatment option of AD is improvement of brain cholinergic function by the use of acetylcholinesterase inhibitors [17-19]. AChE inhibitors are also used for the treatment of imbalance and other related symptoms of Parkinson's disease [20, 21]. However, the currently available drugs used for AChE inhibition have side effects [22].

Glucuronidation helps in detoxification of an organism. β-Glucuronidases hydrolyze glycosidic bonds connecting glucuronic acid with toxic molecules present in the living organisms thus preventing excretion of toxic molecules. So, β-Glucuronidase enzymes present in lysosomes prevent detoxification process causing different diseases. β-Glucuronidase inhibition is suggested to be an important strategy to prevent diseases associated with hepatic disorders, colonic carcinogenesis, and hormone dependent cancers of breast and prostate [23, 24].

Edible leafy vegetables are included in diet of human beings for ages. Most of the reports emphasized on minerals and other nutritional parameters [25-30]. Previously Dasgupta and De [31] reported antioxidant activity of some Indian leafy vegetables. Phytochemicals present in plant- based diets play important roles in controlling different diseases [32]. There are few [33, 34] reports regarding characterization of phytochemical composition of leafy vegetables. Characterization of such phytochemicals is essential. So, attempts were made to screen potential of some leafy vegetables of India in inhibiting enzymes related to stress induced disorders like diabetes or hyperglycemia, hyperlipidemia, liver disorders, AD and to compare their phytochemicals by GC- MS and HPLC based analysis.

Materials and methods Plant material

Leafy vegetables were obtained from the local market of Kolkata and surrounding areas and identified botanically. Extracts of leafy parts of the plants that were studied for their bioactivity and chemical profiling, were *Bacopa monnieri* (BM), *Bauhinia acuminata* (BA), *Centella asiatica* (CA), *Chenopodium album* (ChA), *Coriandrum sativum* (CS), *Enhydra fluctuans* (EF), *Ipomoea aquatica* (IA), *Hygrophila schulli* (HS), *Marsilea minuta* (MM), *Mollugo stricta* (MS) *Moringa oleifera* (MO), *Nyctanthes arbortristis* (NA), *Paederia foetida* (PF), and *Trigonella foenum- graecum* (TF).

Extraction of plant materials

The dried plant materials were extracted by reflux method with 100% methanol for 5 hrs [35]. The filtered extracts were evaporated to dryness and stored at -20oC.

Determination of α-amylase inhibitory activity Inhibitory property of crude extracts against the enzyme α-amylase was studied following the modified method of Bernfeld [36]. Each reaction mixture consisted of α-amylase [ex-porcine pancreas; 0.2 ml of 0.003% solution dissolved in 0.02 M phosphate buffer; pH 6.9], and 0.1 ml of extract. The mixture was incubated for 20 min at 37oC. After that, 0.1 ml soluble starch solution in 0.02 M phosphate buffer was added. The mixture was then incubated again at 37oC for 3 min. Dinitrosalicylic acid reagent (0.2 ml) was added. The mixture was then heated for 5 min in boiling water bath, cooled, diluted by adding 4 ml distilled water. The optical density was measured at 540 nm.

Determination of α-glucosidase inhibitory activity

The α-glucosidase inhibition property was studied using the substrate *p*-nitrophenyl-α-D- glucopyranoside [37]. The reaction mixture consisting of α-glucosidase (ex-microorganism) [0.13 ml of 0.006% solution prepared in 0.02 M phosphate buffer, pH 6.3], plant extract (0.13 ml) and 0.02 M phosphate buffer (0.45 ml) was incubated at 25oC for 1hr. Then 2 M p-nitrophenyl-α-D- glucopyranoside (0.67 ml) was added. The mixture was incubated again for 30 min at 30oC. The reaction was terminated by adding 1 M Na2Co3 solution. Optical density was measured at 405 nm. The percent enzyme inhibition activity was calculated.

Determination of acetylcholinesterase inhibitory activity

Acetylcholinesterase (AChE) obtained from electric eel, methanolic solution of plant extract, and buffer were mixed [38]. The reaction was started by adding 5.5′-dithiobis-(2-nitrobenzoic acid) and acetylthiocholine iodide solution. The reaction mixture was incubated at 37 °C for 20 min. Optical density of the reaction mixture was read at 412 nm.

Determination of lipase inhibitory activity

The reaction mixture consisted of tris-buffer (400 ml), lipase (ex-porcine pancreas; 150 µl), extract solution (50 µl), and p-nitrophenyl laureate (450 µl) [39]. The mixture was incubated for 2 hrs. at 37 °C. After centrifugation, the optical density was measured at 400 nm.

Determination of β-glucuronidase inhibitory activity

β-Glucuronidase inhibition activity was measured with little modification of Kim *et al*. [40] The reaction mixture consisted of β-glucuronidase (ex-Bovine liver; 100 µl of 986.4 units / ml solution in 0.1 M phosphate buffer, pH 7.0), extract (340 µl solution in 0.1 M phosphate buffer). Following preincubation for 15 min. at 37 °C, *p*-nitrophenyl-βD glucuronide solution (60 µl; 3.15 mg/ml in 0.1 M phosphate buffer, pH 7.0 was added and the mixture was again incubated at 37oC for 50 min. The colour developed was read at 405 nm.

Determination of IC50 values

IC50 values (Extract concentration required for inhibition of enzyme activity by 50%) were calculated from the regression equations prepared from the concentrations of the extracts and the respective percent enzyme inhibition.

Gas chromatography – mass spectrometric (GC-MS) analysis

Sample of dried extract was dissolved in methanol (2 ml). Dissolved sample was warmed for a while on a boiling water bath. Adonitol (20 µl) was added. The extract solution (200µl) was evaporated to dryness. The residue, redissolved in 10 µl of methoxyamine hydrochloride solution (20 mg/ml pyridine) was shaken for 90 min at 30 °C. Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (90 µl) was added. The mixture was shaken at 37OC for 30 min for trimethylsilylation. Fatty acid methyl esters (2 µl) were added. [41] GC-Ms parameters were mentioned previously [21, 22].

HPLC analysis

The HPLC analyses were carried out by an Agilent 1260 infinity series HPLC system comprised of a quaternary pump with vacuum degasser and a diode array detector (DAD). The chromatographic conditions included Waters Symmetry reverse phase C18 column [dimension: 150 nm x 4.6 (id) and 5□m particle size]. The ultra violet detection wavelength for both rutin and quercetin was set at 254 nm for qualitative analyses [42]. The metabolites were eluted by the mobile phase containing aqueous formic acid (0.1%) [A] and acetonitrile containing 0.1% formic acid [B]. The ratio of A: B (v/v) was 90:1 linearly changed to 55:45 for 30 min, to 10:90 after 30 min, and kept at 10: 90 from 30 to 40 min. The flow rate was set at 0.5 ml/min.

Determination of total phenol content

Total phenol content was measured by Folin-ciocalteau reagent and was expressed as gallic acid equivalent (GAE) [43].

Determination of total flavonoid content

Total flavonoid content was measured following Kim *et al.* [44] and was expressed as catechin equivalent (CE).

Results and Discussion

α-Glucosidase inhibition activity

Activities of methanol extracts of all leafy vegetables (concentration up to 1.5 mg/ml) were examined against α-glucosidase. All samples showed good activity in a concentration dependent manner. Comparison of IC50 values between different extracts of leafy vegetables is exhibited in Fig. 1. Highest activity, as determined by the IC50 values, was found in *Bacopa monnieri* (BM). *Coriandrum sativum* (CS) recorded lowest activity.

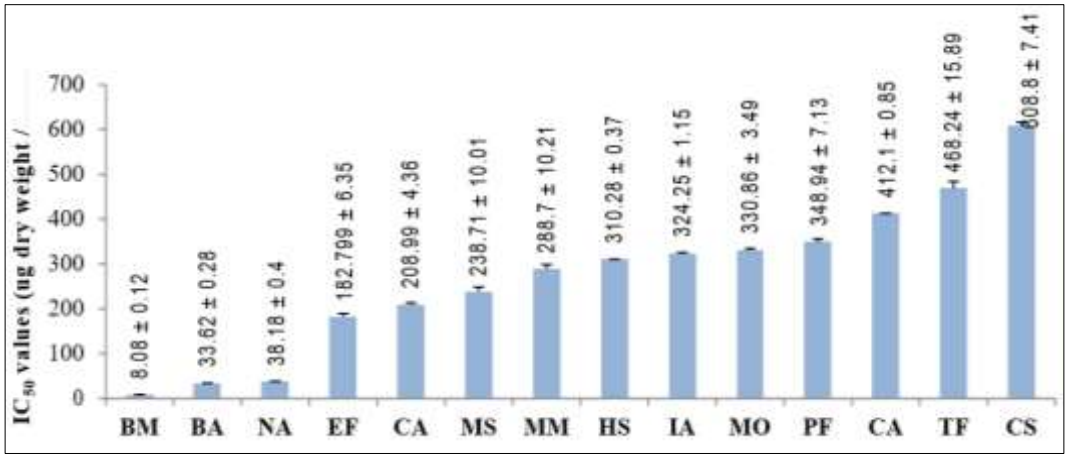

Fig 1: α-Glucosidase inhibitory activity of leafy vegetables (methanolic extract)

α-Amylase inhibitory activity

The activity of different concentrations (up to 0.7 mg/ml) of the extracts of leafy vegetables were examined against α-amylase. Except *Bauhinia acuminata* leaf (White flowered variety), other vegetables had no activity up to the tested concentration. *Bauhinia acuminata* leaf showed good concentration dependent α-amylase inhibitory activity (Fig. 2). IC50 value of *Bauhinia acuminata* leaf (white flowered variety) (465.57±8.81 µg/ ml) was higher than the IC50 value (0.006 µg/ml) of acarbose [45], indicating significantly lower activity.

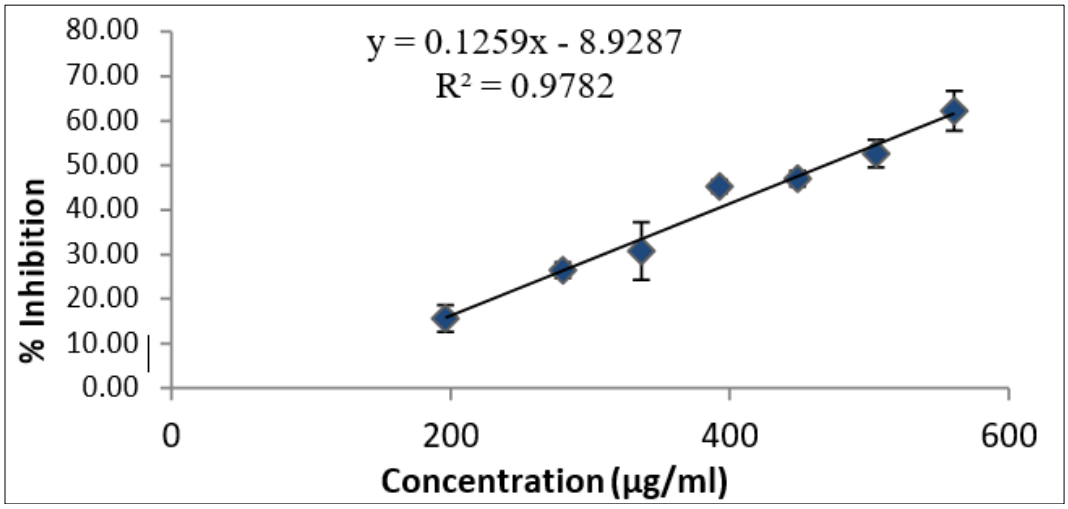

Fig 2: α-Amylase inhibitory activity of Bauhinia acuminata leaf (white flowered variety)

Lipase inhibitory activity

Present findings reveal that the methanol extracts of the leafy vegetables inhibited pancreatic lipase in a concentration dependent manner. IC50 values (Fig. 3) for inhibition of the enzyme lipase by the extracts IA, NA, MO, CA and HS were found to be significantly lower than that of orlistat (IC50 value = 153.74±6.5 µg/ml), indicating stronger activity. Present comparative study revealed that *Ipomoea aquatica*

(IA) was the most potential to inhibit pancreatic lipase. Consumption of edible plants could be a more effective method for the prevention or treatment of hyperlipidemia [12]. However, the activity of PF, TF, and EF was lower than that of orlistat. Thus, the methanol extracts of the presently studied leafy vegetables have potential lipase inhibitory properties which may be good for controlling obesity.

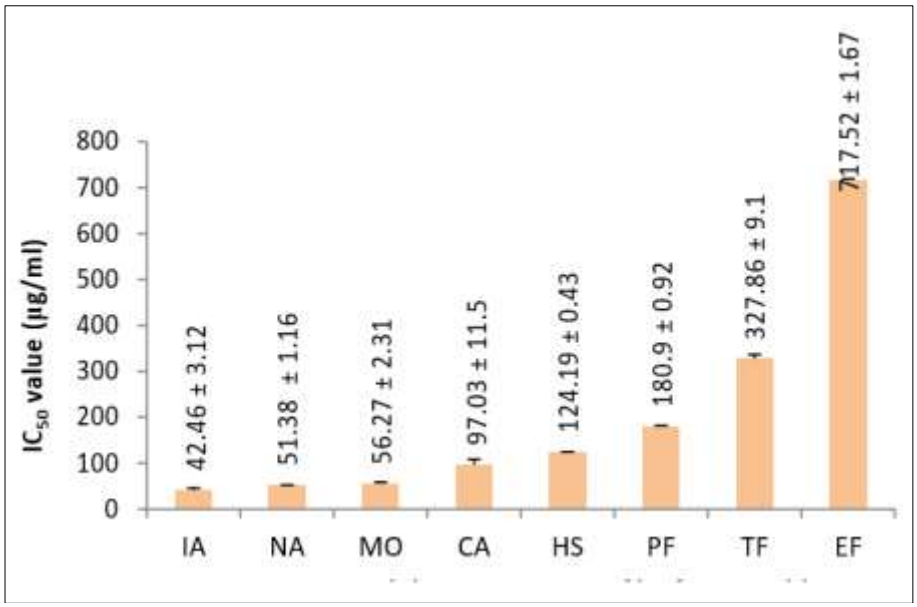

Fig 3: Lipase inhibitory property of leafy vegetables

Acetylcholinesterase inhibitory activity

Methanolic extracts of the leafy vegetables were examined for their AChE inhibitory activities. Highest activity was observed in NA followed by IA, BA, HS, and TF (Fig. 4). Methanolic extracts of the other vegetables did not show activity. Galanthamine is a plant derived alkaloid commercially available as inhibitor of acetylcholinesterase [46] The IC50 values of the active extracts were compared to that of galanthamine (IC50 value 8.243±0.016 µg/ml or 22.38±0.44 µM) [22]. All the extracts showed activities much lower than that of galanthamine.

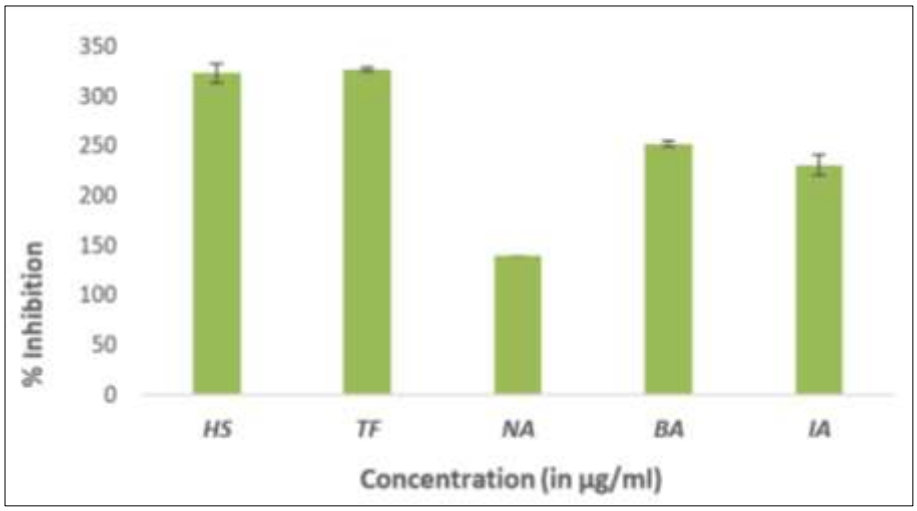

Fig 4: Comparison of acetylcholinesterase inhibitory activity of leafy vegetables

β-Glucuronidase inhibitory activity

Concentration required for 50% inhibition of enzyme activity (IC50 value) for silymarin, the known glucuronidase inhibitor, was detected to be 0.79±0.01 mg/ml [47]. So, glucuronidase inhibition properties of the extracts of all the leafy vegetables were compared at 500 µg (dry weight of extract) / ml concentration. Percent enzyme inhibition activity of the extracts has been compared in Fig. 5. ChA, CA, MO did not show any activity at the concentration tested. Activity of BM was not measured. At 500 µg / ml concentration, crude extracts inhibited the enzyme activity by more than 50%. This suggested that the extracts had activity higher than that of silymarin. It has previously been reported that human serum glucuronidase activity was inversely associated with consumption of plant-based food [48]. Present study shows that at 500 µg / ml concentration the extracts of CS, EF, HS, IA, NA, PF and TF inhibited the enzyme activity by more than 50%. This suggested that the extracts had activity higher than that of silymarin.

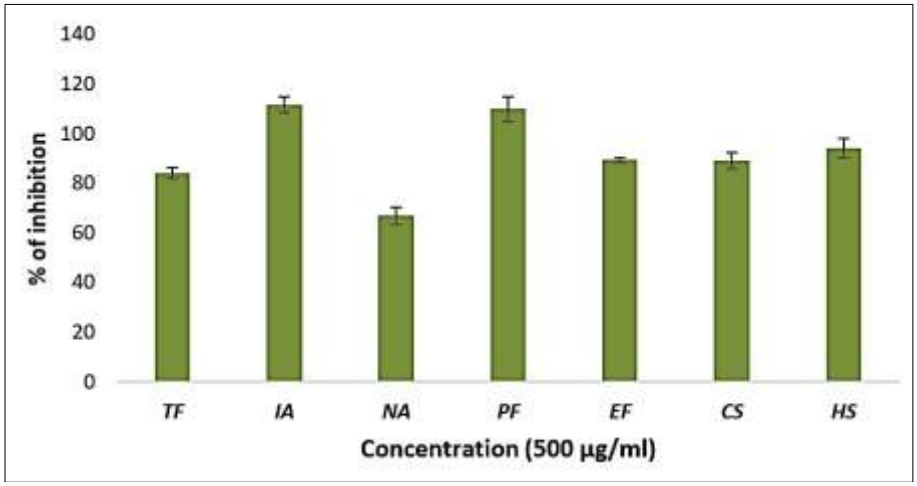

Fig 5: Comparison of β-glucuronidase inhibitory activity of leafy vegetables

Enzyme inhibitory properties of phenolic compounds

Total phenol content and total flavonoid content of methanolic (Fig. 6) extracts were also measured. Methanolic extract of BM had highest total phenol content followed by NA, BA, HS, EF, MS, PF, TF, IR, CS, CA, MO, MM, and ChA.

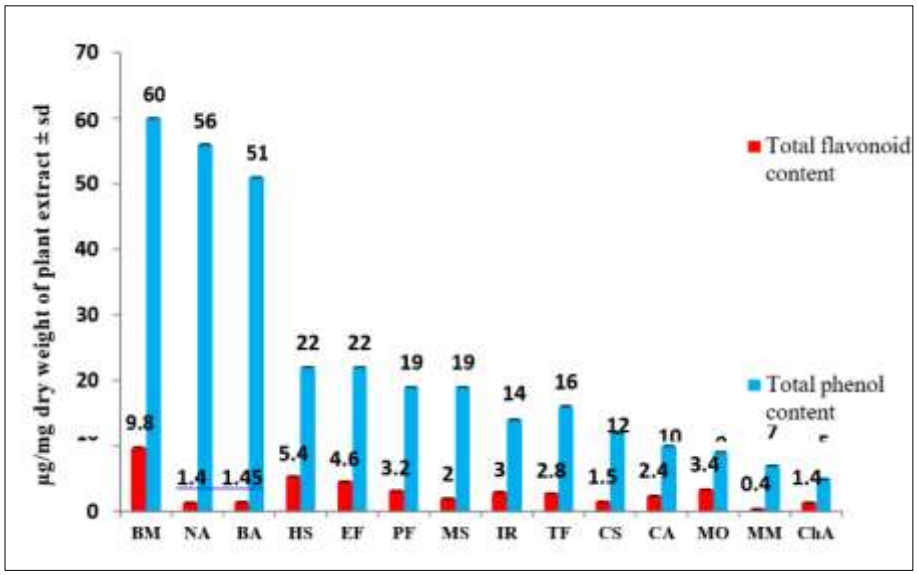

Fig 6: Total flavoniod content and total phenol content of extracts

A-glucosidase inhibitory activity of the extracts was well co-related with the total phenol content (R2 = 0.79) and total flavonoid content (R2 = 0.8). Total phenol content was inversely proportional to the IC50 values of the methanolic extracts of plant materials suggesting possible involvement of the phenols and flavonoids in the activity. Synergistic activity is also a possibility.

Table 1: Phenolic compounds identified from leafy vegetables

Phenolsidentified / Leafy vegetables	***Coriandrum sativum***	***Ipomoea aquatica***	***Paederia foetida***	***Trigonella foenum-graecum***	***Centella asiatica***	***Chenopodium album***	***Enhydra fluctuans***	***Hygrophila schulli***	***Marselia minuta***	***Moringa oleifera***	***Nyctanthes arbortristis***	**Reported inhibitory activities of metabolites against enzymes**
	CS	**IA**	**PF**	**TF**	**CA**	**ChA**	**EF**	**HS**	**MM**	**MO**	**NA**	
Phenols detected by GC-MS												
Benzoic acid	+		+	+	+	+	+	+		+	+	α-Amylase [49]
Resorcinol	+		+								+	
Hydroquinone			+				+				+	
O-Acetylsalicylic acid		+	+	+		+	+	+			+	β-Glucuronidase [47]
Pyrogallol											+	
Benzene-1,2,4-triol											+	AChE [22] α-Amylase [50]
4-Hydroxybenzoic acid	+	+	+	+	+	+	+	+	+	+	+	Lipase [51]
Phloroglucinol	+										+	
2,3-Dihydroxybenzoic				+								

acid												
4-Hydroxy-3-methoxy benzoic acid (vanillic acid)	+	+	+									
Shikimic acid	+	+	+	+	+		+	+	+	+	+	
3,4-Dihydroxybenzoic acid (protocatechuic acid)	+	+	+				+	+	+	+		α-Amylase [49] α-Glucosidase [49]
Quinic acid	+	+	+		+		+		+	+		α-Amylase [49] α-Glucosidase [49] β-Glucuronidase [47]
4-Hydroxycinnamic acid (*p*-Coumaric acid)	+	+	+				+		+			α-Glucosidase [48]
Gallic acid		+	+			+						AChE [52, 53] Lipase [51] α-Amylase [50. 54] α-Glucosidase [50, 54]
Ferrulic acid		+	+		+			+				α-Glucosidase [49]
Caffeic acid	+	+	+		+		+	+	+		+	α-Amylase [49] α-Glucosidase [49]
Arbutin	+											
Neohesperidin	+							+		+		
Epicatechin	+											β-Glucuronidase [55] Lipase [54]
Catechin							+					α-Amylase [54]
Diadzein	+				+							
Taxifolin					+							α-Amylase [49]
Kaempferol	+		+	+	+				+		+	AChE [52] β-Glucuronidase [56]
Chlorogenic acid	+	+	+		+				+			β-Glucuronidase [55] α-Amylase [50] α-Glucosidase [50]
Isoquercitrin	+		+				+					
Hesperetin			+									
HPLC identified phenols												
Rutin	+	+	+	+	+	+		+	+	+	+	
Quercetin	+	+	+	+	+	+		+	+	+	+	

Apart from the nutrients such as minerals, vitamins, carotenoids etc., vegetables are also sources of different phytochemicals produced by plants. Different such phytochemicals, particularly the phenols, are bioactive compounds which have medicinal and disease preventing properties. So, we also compared the phenolic profile of the leafy vegetables used during this study (Table 1). It appears that the plants contain different compositions of phenols in different plants. Many of such metabolites have been found to have enzyme inhibitory properties as reported earlier from the laboratory (Table 1). In comparison to pharmaceutical drugs, bioactive phytochemicals have low potency in plants. But these bioactive phytochemicals are consumed regularly in good amounts as diet. So regular consumption of plant-based diet may have significant physiological effect. [57] Some metabolites, not identified, may also have contribution to the enzyme inhibitory activities of extracts of the leafy vegetables. So, addition of more leafy vegetables in diet may be beneficial in combating different diseases.

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