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EFFECT OF SOLVENT EXTRACTION SYSTEM ON THE ANTIOXIDANT ACTIVITY OF SOME SELECTED WILD LEAFY VEGETABLES OF MEGHALAYA STATE IN INDIA

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ABSTRACT: The antioxidant activities of five wild leafy vegetables e.g. *Allium porrum*, *Carpesium cernuum*, *Tricyrtis pilosa*, *Spilanthes acmella*, and *Leea sambucina* collected from Meghalaya state in India were determined by using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, reducing power ability, estimation of total phenolic content, flavonoid content and flavonol content.. The results indicate that these wild edible plants can be utilized as natural antioxidant. The solvent systems used were benzene, chloroform, acetone and methanol. The different levels of antioxidant activities were found in the solvent systems used. The results indicate that these wild edible vegetables can be utilized as natural antioxidant.

INTRODUCTION: An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. As antioxidants have been reported to prevent oxidative damage caused by free radical, it can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers [1]. Reactive oxygen species affect living cells and these radicals are responsible to many chronic diseases in humans such as atherosclerosis, parkinson's disease, arthritis, alzheimer's disease, stroke, chronic inflammatory diseases, cancers, and other degenerative diseases [2].

Plant materials are the rich source of active constituents of varied chemical characteristics and polarities and complete extraction of active components, responsible for antioxidant activities, are strongly dependant on the nature of solvents and plant parts used. During the extraction of plant material, the selection of solvents and plant parts is very much important to minimize interference from compounds that may co-extract with the chemicals and to avoid the contamination of the extract.

Solvents, such as methanol, ethanol, acetone, chloroform and ethyl acetate have been widely used for the extraction of antioxidant compounds from various plants and plant based foods and medicines. Results of previous studies showed that the extraction yield of phenolic and flavonoid content is greatly depending on the polarity of the solvent [3-4].

From literature survey, it is reported that maximum phenolic compounds were obtained from barley flour with the mixture of ethanol and acetone [5]. The aq methanol was found to be more effective solvent to extract the phenolic compounds from rice bran and *Moringa oleifera* leaves [6-7]. The extraction of high content of antioxidant compounds with 80 % Aq. methanol (methanol: water 80 : 20) were found from various plant materials like rice bran, wheat bran, oat groats and hull, coffee beans, citrus peel and guava leaves as reported by Anwar *et al*., 2006 [8]. The highest extract yields were obtained from polar alcohol based solvents. Addition of water to ethanol improves the extraction rate but too high water content may leads to the extraction of other compounds.

The highest level of phenolic compounds was found with 50% acetone from wheat, whereas ethanol is the least effective solvent to isolate phenolics from wheat bran [9]. It can be concluded that it is not clear which type of solvent is more effective for extracting the antioxidant components from plant.

Therefore, the objective of present study was to investigate the effect of different extracting solvents with different polarity on the antioxidant activities of five leafy vegetables from North-East India viz *Allium porrum*, *Carpesium cernuum*, *Tricyrtis pilosa*, *Spilanthes acmella* and *Leea sambucina*. Thus the results from this preliminary study will provide a better understanding of the antioxidant properties of these plants and would be enabling to develop natural antioxidant.

MATERIALS AND METHODS:

Plant materials : The five plant materials e.g the leaves of *Allium porrum*, *Carpesium cernuum*, *Tricyrtis pilosa*, *Spilanthes acmella*, and *Leea sambucina* were collected from different market of Meghalaya state, India on March 2011 and authenticated in our office. The voucher specimens were preserved in the Plant Chemistry department of our office under registry no BSITS 36, BSITS 37, BSITS 38, BSITS 39, BSITS 40 respectively. The plant parts were shed-dried, pulverized and stored in an airtight container for further extraction.

Chemicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid, quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA)., Folin-Ciocalteus's phenol reagent, gallic acid, potassium ferricyanide, Aluminium chloride, $FeCl_3$ and sodium carbonate were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used including the solvents, were of analytical grade.

Extraction of plant material (Benzene, chloroform, Acetone and Methanol): One gram of each plant material were extracted with 20 ml each of benzene, chloroform, acetone and methanol with agitation for 18 -24 h at ambient temperature. The extracts were filtered and diluted to 50 ml and aliquot were analyzed for their total phenolic, flavonoid and flavonol content, reducing power and their free radical scavenging capacity.

Estimation of Total Phenolic Content : The amount of total phenolic content of crude extracts was determined according to Folin-Ciocalteu procedure [10]. 20 - 100 µl of the tested samples were introduced into test tubes. 1.0 ml of Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured (UV-visible spectrophotometer Hitachi U 2000 Japan). The total phenolic content was expressed as gallic acid equivalents (GAE) in miligram per gram (mg/g) of extract using the following equation based on the calibration curve;

$$y = 0.0013x + 0.0498, \; R^2 = 0.999$$

where y was the absorbance and x was the Gallic acid equivalent (mg/g).

Determination of Total Flavonoids : Total flavonoids were estimated using the method of Ordonez *et al*., 2006 [11]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour, at room temperature, the absorbance was measured at 420 nm (UV-visible spectrophotometer Hitachi U 2000 Japan). A yellow color indicated the presence of flavonoids. Total flavonoid contents were calculated as rutin (mg/g) using the following equation based on the calibration curve:

$$y = 0.0182x - 0.0222, \; R^2 = 0.9962$$

where y was the absorbance and x was the Rutin equivalent (mg/g).

Determination of Total Flavonols : Total flavonols in the plant extracts were estimated using the method of Kumaran and Karunakaran, 2006 [12]. To 2.0 ml of sample (standard), 2.0 ml of 2% $AlCl_3$ ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm (UV-Visible Spectrophotometer Hitachi U 2000 Japan) was read after 2.5 h at 20°C. Total flavonol content was calculated as quercetin (mg/g) using the following equation based on the calibration curve:

$$y = 0.0049x + 0.0047, \; R^2 = 0.9935$$

where y was the absorbance and x was the quercetin equivalent (mg/g).

Measurement of Reducing Power: The reducing power of the extracts was determined according to the method of Oyaizu, 1986 [13]. Extracts (100 µl) of plant extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, *p*H 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of 10% trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Reducing power is given in ascorbic acid equivalent (AAE) in milligram per gram (mg/g) of dry material using the following equation based on the calibration curve:

$$y = 0.0023x - 0.0063, R^2 = 0.9955$$

where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

Determination of Free Radical Scavenging Activity: The free radical scavenging activity of the plant samples and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) [14].

Aliquots (20 - 100 µl) of the tested sample were placed in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and mixed. 30 min later, the absorbance was measured at 517 nm (UV-visible spectrophotometer Hitachi U 2000 Japan). The capability to scavenge the DPPH radical was calculated, using the following equation:

$$\text{DPPH scavenged (\%)} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material per ml (mg / ml) that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation. Values are presented as mean ± standard error mean of three replicates. The total phenolic content, flavonoid content, flavonol content, reducing power and IC_{50} value of each plant material was calculated by using Linear Regression analysis.

RESULTS AND DISCUSSION:

Extractive value: The extractive value of the tested wild edible leafy vegetables with four different solvents are depicted in **Table 1**. The yield of extracts using benzene, chloroform, acetone and methanol in case of leaves of *A. porrum* were 1.85±0.03, 3.67±0.09, 2.88± 0.01, 15.90±0.06 g/100g dry material respectively. Likewise the leaf extract of other plant materials also followed the same order as *A. porrum* extracts. The differences in the extractive value of the plants may be due to the varying nature of the components present and the polarities of the solvent used for extraction [15].

Total Phenol, Flavonoid and Flavonol content of the Extracts: The screening of the benzene, chloroform, acetone and methanol extracts of five wild plants revealed that there was a wide variation in the amount of total phenolics ranging from 2.68±0.58 to 75.34 ±1.00 mg GAE/g dry materials (**Table 2**). The highest amount of phenolic content was found in the methanol extract of *L. sambucina* (75.34±1.00 mg GAE/g dry material) followed by the methanol extract of *S. acmella* (36.43±0.48 GAE) **.** While lower amounts was observed in the chloroform extract of *C. cernuum* (2.68 ±0.58 GAE). The methanol extracts of *T. pilosa* (32.61 ±0.88 GAE) and C. *cernuum* (25.53±0.88 GAE) were also found to contain a very good amount of phenolic compounds.

The flavonoid contents of the extracts in terms of rutin equivalent were between 13.52±0.17 to 284.60±0.03 mg/g dry material (**Table 3**). The highest amount of flavonoid was found in the chloroform extract of *A. porrum* and the benzene and acetone extract of this plant also contain a very good amount of flavonoids. The benzene, chloroform and acetone extract of *T. pilosa* also contain a very good amount of flavonoids.

The flavonol contents in the different extracts of plant materials were evaluated in terms of quercetin equivalent (**Table 4**). The highest amount of flavonol was observed in the chloroform extract of *T. pilosa* (230.82±2.79 mg/g). The other extracts of this plant also contain a very good amount of flavonol. Very good amounts of flavonol were also found in the benzene, chloroform and acetone extract of *A. porrum* and *C. cernuum*.

It has been established that phenolic compounds are the major plant compounds with antioxidant activity and this activity is due to their redox properties. Phenolic compounds are a class of antioxidant agents which can adsorb and neutralize the free radicals [16]. Flavonoids and flavonols are regarded as one of the most widespread groups of natural constituents found in the plants. It has been recognized that both flavonoids and flavonols show antioxidant activity through scavenging or chelating process [17].

The results strongly suggest that phenolics are important components of these plants. The other phenolic compounds such as flavonoids, flavonols, which contain hydroxyls are responsible for the radical scavenging effect in the plants.

According to our study, methanol was the most suitable solvent to isolate the phenolic compounds and benzene, chloroform and acetone are the best solvent to isolate the flavonoids and flavonols from the plant materials. The high content of the phenolic compounds in *L. sambucina*, *S. acmella, T. pilosa* and *C. cernuum* can explain their high radical scavenging activity.

Reducing Power Assay: The reducing powers of the five wild leafy vegetables were evaluated as mg AAE/g dry material as shown in **Table 5**. The highest reducing power was exhibited by the benzene extract of *T. pilosa* (256.52±3.59 mg/g AAE) which also contain a very good amount of flavonoids and flavonols. The methanol extract of *A. porrum* showed lowest activity in terms of ascorbic acid equivalent (12.07±0.44 mg/g AAE). In this assay, the presence of antioxidants in the extracts reduced Fe^{+3}/ferricyanide complex to the ferrous form. This reducing capacity of the extracts may serve as an indicator of potential antioxidant activities through the action of breaking the free radical chain by donating hydrogen atom [18].

DPPH Radical Scavenging Activity: The evaluation of anti-radical properties of five wild leafy vegetables were performed by DPPH radical scavenging assay. The 50% inhibition of DPPH radical (IC_{50}) by the different plant materials was determined (**Table 6**), a lower value would reflect greater antioxidant activity of the sample. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [19].

The antioxidant effect is proportional to the disappearance of the purple colour of DPPH in test samples. Thus, antioxidant molecules can quench DPPH free radicals by providing hydrogen atom or by electron donation and a colorless stable molecule 2, 2-diphenyl-1-hydrazine is formed and as a result of which the absorbance (at 517 nm) of the solution is decreased.

TABLE 1: EXTRACTIVE VALUE OF LEAFY VEGETABLES COLLECTED FROM MEGHALAYA USING DIFFERENT SOLVENTS

Sl. No.	Name of the plant	Parts used	Extractive value (g / 100g dry material)			
			Benzene	Chloroform	Acetone	Methanol
1	*Allium porrum*	leaves	1.85±0.03	3.67±0.09	2.88±0.01	15.90±0.06
2	*Carpesium cernuum*	leaves	1.03±0.03	2.20±0.06	2.000.06	5.10±0.06
3	*Tricyrtis pilosa*	leaves	1.18±0.01	2.67±0.01	3.83±0.01	8.34±0.03
4	*Spilanthes acmella*	leaves	1.20±0.06	2.45±0.03	1.80±0.03	9.10±0.06
5	*Leea sambucina*	leaves	1.60±0.06	1.65±0.03	1.50±0.06	4.40±0.06

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

TABLE 2: TOTAL PHENOLIC CONTENT IN THE LEAFY VEGETABLES COLLECTED FROM MEGHALAYA USING DIFFERENT SOLVENTS

Sl. No.	Name of the plant	Parts used	Total phenolic content (GAE mg / g dry material)			
			Benzene	Chloroform	Acetone	Methanol
1	*Allium porrum*	leaves	3.10 ±0.67	17.43 ±0.36	17.50 ±0.76	5.93 ±0.42
2	*Carpesium cernuum*	leaves	5.25 ±0.64	2.68 ±0.58	15.76 ±1.11	25.53 ±0.88
3	*Tricyrtis pilosa*	leaves	15.01 ±1.83	9.77 ±0.80	10.70 ±1.93	32.61 ±0.88
4	*Spilanthes acmella*	leaves	4.38 ±0.53	3.38 ±0.88	6.12 ±1.88	36.43 ±0.48
5	*Leea sambucina*	leaves	5.28 ±1.38	6.08 ±0.80	15.04 ±1.70	75.34 ±1.00

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

TABLE 3: TOTAL FLAVONOID CONTENT IN THE LEAFY VEGETABLES COLLECTED FROM MEGHALAYA USING DIFFERENT SOLVENTS

Sl. No.	Name of the plant	Parts used	Total flavonoid content (Rutin eqivalent mg / g dry material)			
			Benzene	Chloroform	Acetone	Methanol
1	*Allium porrum*	leaves	159.90±1.00	284.60±0.03	132.20±1.56	62.72±0.03
2	*Carpesium cernuum*	leaves	59.85±0.51	64.46±1.89	53.28±0.36	26.37±0.20
3	*Tricyrtis pilosa*	leaves	245.05±1.63	152.08±2.00	91.30±0.50	41.60±0.75
4	*Spilanthes acmella*	leaves	36.37±0.20	55.85±0.82	40.83±0.50	22.97±0.12
5	*Leea sambucina*	leaves	23.84±0.21	30.60±0.10	22.56±0.38	13.52±0.17

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

TABLE 4: TOTAL FLAVONOL CONTENT IN THE LEAFY VEGETABLES COLLECTED FROM MEGHALAYA USING DIFFERENT SOLVENTS

Sl No	Name of the plant	Parts used	Total flavonol content (Quercetin eqivalent mg / g dry material)			
			Benzene	Chloroform	Acetone	Methanol
1	*Allium porrum*	leaves	182.76±1.24	190.17±0.61	142.85±1.32	41.65±1.01
2	*Carpesium cernuum*	leaves	168.67±2.12	165.57±0.31	178.55±1.51	73.12±1.59
3	*Tricyrtis pilosa*	leaves	201.07±2.57	230.82±2.79	102.01±2.58	66.93±1.19
4	*Spilanthes acmella*	leaves	54.68±0.98	77.40±0.49	70.65±2.63	26.46±0.60
5	*Leea sambucina*	leaves	78.21±0.77	90.97±1.74	46.46±1.42	28.98±1.24

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

TABLE 5: REDUCING POWER (ASCORBIC ACID EQUIVALENT) OF THE LEAFY VEGETABLES COLLECTED FROM MEGHALAYA USING DIFFERENT SOLVENTS

Sl. No	Name of the plant	Parts used	Reducing power (Ascorbic acid eqivalent mg / g dry material)			
			Benzene	Chloroform	Acetone	Methanol
1	*Allium porrum*	leaves	37.34±1.66	19.44±0.62	34.96±1.80	12.07±0.44
2	*Carpesium cernuum*	leaves	20.94±1.92	43.77±2.61	90.18±3.78	46.65±1.26
3	*Tricyrtis pilosa*	leaves	256.52±3.59	178.35±3.95	161.88±1.67	84.03±0.77
4	*Spilanthes acmella*	leaves	58.51±3.14	69.25±1.05	127.17±2.09	28.34±0.56
5	*Leea sambucina*	leaves	62.91±2.35	125.41±3.59	164.20±1.45	56.97±2.54

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

TABLE 6: FREE RADICAL SCAVENGING ABILITY OF THE LEAFY VEGETABLES COLLECTED FROM MEGHALAYA BY THE USE OF A STABLE DPPH RADICAL (ANTIOXIDANT ACTIVITY EXPRESSED AS IC $_{50}$)

Sl. No.	Name of the plant	Parts used	Free radical scavenging ability (IC_{50} mg / g dry material)			
			Benzene	Chloroform	Acetone	Methanol
1	*Allium porrum*	leaves	0.580±0.105	0.55±0.10	0.69±0.04	0.43 ±0.0028
2	*Carpesium cernuum*	leaves	0.205±0.003	0.232±0.026	0.208±0.003	0.129±0.006
3	*Tricyrtis pilosa*	leaves	0.320±0.034	0.283±0.014	0.145±0.004	0.128±0.0027
4	*Spilanthes acmella*	leaves	0.339±0.018	0.388±0.032	0.222±0.005	0.18±0.0011
5	*Leea sambucina*	leaves	0.564±0.058	0.219±0.017	0.134±0.001	0.12±0.0013

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

Hence, the more potent antioxidant, more decrease in absorbance is seen and consequently the IC_{50} value will be minimum. In the present study, the highest radical scavenging activity was shown by the methanol extract of *L. sambucina* (IC_{50} = 0.12±0.0013 mg dry material), whereas the acetone extract of *A. porrum* showed lowest activity (IC50 = 0.69±0.04 mg dry material). Strong inhibition was also observed for the methanol extract of *S. acmella* (IC_{50} = 0.18±0.0011 mg dry material, *T. pilosa* (0.128±0.0027 mg dry material) and *C. cernuum* (IC_{50} = 0.129±0.006 mg dry material).

The high radical scavenging property of *L. sambucina* may be due to the hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as a radical scavenger.

The benzene, chloroform, acetone and methanol extracts of all of the leafy vegetables under investigation exhibited different extent of antioxidant activity and thus provide a valuable source of nutraceutical supplements.

CONCLUSION: The result of present study showed that the methanol extract of *L. sambucina*, which contain highest amount of phenolic compounds exhibited the greatest radical scavenging activity. The benzene, chloroform and acetone extract of *A. porrum, S. acmella, T. pilosa* and *C. cernuum* contain highest amount of flavonoids and flavonols also showed strong radical scavenging activity. The radical scavenging activities of the selected plants extracts are still less affective than the commercial available synthetic like BHT. As the plant extracts are quite safe and the use of synthetic antioxidant has been limited because of their toxicity, therefore, these wild edible plants could be exploited as antioxidant additives or as nutritional supplements.

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