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RESEARCH ARTICLE

ANALYSIS OF CHEMICAL CONSTITUENTS, PHYTOCHEMICAL CONTENT AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *TRICHOSANTHES DIOICA* PLANT LEAF (LESS FOCUSED VALUABLE MEDICINAL HERB) EXTRACT

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ABSTRACT

The ancient use of *Trichosanthes dioica* leaves as a valuable remedy in traditional medicine guided the authors to search out for its inherent chemical characteristics responsible for such activity. The present study encompasses a complete analysis of the leaf portion W.R.T. protein, carbohydrate, moisture, vitamin C, minerals, phytochemicals and in-vitro antioxidant activity in term of DPPH and $ABTS^+$ free radical scavenging assay. Experimental data of the leaves were expressed on the basis of a comparative analysis w.r.t. the corresponding *Trichosanthes dioica* fruits, the data of which were already established. The principal components in general and the carotenoids in particular of the *T. dioica* leaf may place it at a higher position to act as a nutrient pool to fight against Vitamin deficiency. Although *T. dioica* leaves are deficient in sodium, potassium and calcium contents in comparison to the fruits, but in term of % of coparison the high iron content (+5924.12%) and lower phytate content (-70%) make the former a very efficient and economic source of iron nutrition to the anaemic. The higher oxalate (+401.57%) and lower calcium (-28.80%) contents of *T. dioica* leaves make it unsuitable for consumption for the people suffering with Ca-deficiency diseases. Methanolic leaf extract of *T. dioica* is a better source (in term of % of comparison) of polyphenols (+1068.19%) and flavonoids (+664.84%) than the corresponding contents in the fruit part. Result showed that, the extract exhibited significant DPPH (16% more effective than the fruits) and $ABTS^+$ free radical scavenging activities.

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INTRODUCTION

Now-a-days metabolic diseases, degenerative diseases, stress related diseases have created a pressure on modern civilization to think about life style modification, diet, stress relieving factors. Now proper diet, instead of diet, is the most important factor among them because it has the capacity to supply essential nutrients and help to achieve a healthy life. Now, important part of our diet comprises of green leafy vegetables or plant leaves. Green leafy vegetables are the food which has the capacity to supply the essential vitamins, minerals, phytochemicals and natural source of antioxidants. The secondary metabolites of plants, designated as phytochemicals, are naturally occurring and non-nutritional constituents having biological and pharmacological activities, such as antioxidant, anti-inflammatory, antimicrobial, anti allergic, antibiotic, hypoglycaemic etc (Borneo *et al.*, 2008). The most important phytochemicals are phenolic compounds, alkaloids, flavonoids, tannins and saponins. The underlying mechanism of many common and life threatening diseases (atherosclerosis, diabetes, cancer, and aging) involves free radicals (Superoxide anion, Hydroxyl radical, and Peroxy radical), which are either generated from normal metabolism of human body or they are introduced by uv radiation impact and different pollutant induced reactions. Antioxidants are highly efficient to scavenge these free radicals and protect other molecules from oxidation. Plants are not major sources of carbohydrate and protein, but good source of beta-carotene, ascorbic acid (natural antioxidants), vitamin K, calcium, folic acid, riboflavin, iron,

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essential amino acids (Mnzava, 1997 and Fasuyi, 2006). So, due to their nutrient and phytochemical contents, these have the capacity to alleviate or help to alleviate many diseases. Though the modern research has been focused on beneficial effects of many green leafy vegetables, in spite of that there are many plants which have been used by tribal for centuries but scientifically remain unexplored. In modern research it is necessary to find out more and more sources of natural antioxidants, bioactive compounds, neutraceuticals to minimize the side effects of synthetic ones. In that case the plant kingdom is a gold mine. *Trichosanthes dioica* is such herb which has been valued in traditional medicine from time immemorial, but due to lack of scientific awareness of its nutritional parts, it remained less familiar in daily life consumption.

The plants in Cucurbitaceae family is composed of about 110 genera and 640 species (Khare, 2004). Trichosanthes, is an annual or perennial herb distributed in tropical Asia, Polynesia and Australia. Over 20 species are recorded in India of which two, namely T. anguina and T. dioica, are cultivated as vegetables. *Trichosanthes dioica*, commonly known as parwal (Nadkarne, 1982). Leaves are 7.5cm long, ovate oblong, cordate, rigid, rough on both surfaces. The plant has a promising place in Ayurvedic system of medicine from ancient time. According to Ayurveda, leaves of the plant are used as antipyretic, diuretic, cardiotonic, laxative, antiulcer, diabetes mellitus etc (Kumar *et al.,* 2012). In Charaka Samhita, leaves and fruits are used for treating alcoholism, jaundice, edema and alopecia (Khare, 2004). Adiga *et al*. (2010) studied the hypoglycaemic effect of aqueous extract of *Trichosanthes dioica* on normal and diabetic rat. In this study they have observed that the aqueous extract of the leaves lowers serum glucose levels in diabetic rats and significantly increases glucose tolerance. Though scientific work has been done on its hypoglycaemic aspect but nutritional and phytochemical aspects remain unfocused. In spite of having neutraceuticals and potential antioxidant activity, the edible plant leaf remain less focused in our daily diet. Thus, the unfocussed part of *Trichosanthes dioica* leaves insisted the authors to look out for an assay of its nutrient content and antioxidant property, which might be beneficial to ameliorate diseases of the present and future generations.

MATERIALS AND METHOD

Collection and identification of plant materials

Chemicals: 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) stable radical free radical were purchased from Sigma Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.); Ascorbic acid, Trichloroacetic acid (TCA), Ferric Chloride, Anhydrous Sodium Carbonate, Potassium acetate, Potassium permanganate, Aluminum chloride, Butylated Hydroxytoluene, Methanol, Tannic acid, Dichlorophenol-Indophenol dye, Sulphuric acid, Perchloric acid, Nitric acid, Oxalic acid were supplied by E. Merck India Pvt. Ltd. (Kolkata, India). 2,4,6-Tripyridyl-S-Triazine (TPTZ), 2,2 -azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) cation (ABTS +) free radical, Potassium persulfate, Folin Ciocalteu's phenol reagent, Gallic acid and Quercetin were purchased from SRL (Sisco Research Laboratory, Mumbai, India).

Plant Material

The samples were collected from three different districts of West Bengal as well as from three different local markets of Kolkata to obtain the mean value and to exclude the percentage of error as well as possible. The plant materials were identified and authenticated by Botany Department of Calcutta University. The leaves were separated from roots, washed under running water, followed by double glass distilled water. They were drained completely and used for analysis.

Proximate analysis

Proximate composition includes moisture, total ash, crude fibre, fat, protein, carbohydrate and minerals. Moisture was determined by oven dehydration method at 105°C up to the constant weight and expressed in percentage. Protein was determined by estimating the nitrogen content of the plant material using Kjeldahl method. For extraction of total sugar Anthrone-suphuric acid reagent test was applied (Sawhney *et al.,* 2006). For extraction of total lipid, Chloroform:Methanol mixture (2:1) test was applied (Sawhney *et al.*, 2006). Crude fiber was determined by Acid digestion - Alkali digestion and by using fibertech. Ash content was determined in muffle furnace at 550°C for 6 hours. The samples were used in triplicate for all these determinations, according with AOAC14.

Vitamins Assay

Vitamin C was determined in fresh vegetable samples by Dichlorophenol-Indophenol dye reduction method (Sawhney *et al.*, 2006).

Mineral Assay

For minerals analysis viz., K, Ca, Na and Fe- the samples were digested by using $HClO_4$/ HNO_3 method (Steekel *et al.*, 1965). The digested samples were used for selected mineral analysis, using Atomic Absorption Spectrometer (Perkin Elmer model 2380) and Flame Photometer (Jenway PFP7).

Oxalate assay

Total oxalate was analysed by extraction with hydrochloric acid and soluble oxalate with water followed by precipitation with calcium oxalate from deprotinized extract and subsequent titration with potassium permanganate (Baker *et al.*, 1952).

Phytate assay

Phytic acid was extracted and determined according to the precipitate analysis method of Thompson and Erdman 1982 (Thompson *et al.*, 1982) with some modification.

Phytochemicals and *In vitro* antioxidant assay

Preparation of plant leaf extract

After washing the samples were allowed to dry in shades for 3-4 days. After complete drying of this plant leaves, they were

ground into smaller pieces. Plant sample (10g) was mixed with 100ml 80:20 methanol water by maceration for 24 hour. Then the solution was filtered and the filtrates were dried using rotary evaporator under reduced pressure. The extract yields of sample was 10 gm%. Stock solution (1mg/ml) of each plant extract was prepared by using 80% methanol as solvent.

Determination of Total Polyphenol Content (TPC)

Total polyphenol content of the leaf extracts was determined by the method described by Matthaus (2002) with some modification. Briefly, 0.2 ml of different concentrations of the extracts were taken, to which 1ml of Folin-ciocalteu reagent (diluted to 10-folds) and 0.8 ml of 2% Na_2CO_3 were added, and the volume was made up to 10 ml with methanol:water (6:4). After 30 min the absorbance was read at 740 nm wavelength against a standard calibration curve. The results were expressed as Gallic acid equivalents per gram of extract.

Determination of Total Flavonoids Content (TFC)

Total flavonoid content was measured by the aluminium chloride colorimetric assay (Ebrahinzadeh *et al.,* 2008). An aliquot of extracts (50 and 100 μg/ml) or the standard solution of quercetin (20 to 100 µg) were added to a 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml 5% $NaNO_2$ was added. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6^{th} min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with water. The solution was mixed well, and the absorbance was measured against prepared reagent blank at 510 nm.

Determination of Total Hydrolysable Tannin Content (HTC)

Content of tannins was determined by Folin-Denis reagent (Parimala *et al*.,2013) based on colorimetric estimation of tannins (measurement of blue colour formed by the reduction of phosphotungstomolybdic acid by tannins in alkaline medium 0.5gm of dry sample was added to 75 ml of distilled water, heated gently at first and then boiled for 30 minutes. Then it was centrifuged at 2000 rpm for 20 minutes. The supernatant liquid was collected and made up to the volume of 100ml (volumetric flask) by distilled water. After that 0.1ml of this solution was taken in a test tube and 7.5ml of water, 0.5ml of Folin-Denis reagent and 1ml of 35% Na_2CO_3 were added to it. The volume was made up to 10ml (volumetric flask) by using distilled water and shaken well. The test tubes were incubated for 30 minutes at room temperature and the absorbance of the solution was measured at 700nm wavelength against a standard calibration curve. Each experiment was carried out in triplicate and results averaged and expressed as $\pm$SD in µg/100g sample. Tannic acid was used as a standard and the calibration curve was prepared in the range of 20µg-100µg using 20, 40, 60, 80 and 100 µg as the standard concentrations at a wavelength of 700nm (λ_{max} of tannic acid).

***In vitro* Antioxidant activity**

1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging method was used to evaluate the antioxidant property. The antioxidant activity was compared with that of the natural antioxidant, BHT (Butylated hydroxy toluene). Different concentrations of the plant extracts were used to scavenge DPPH. The antioxidant activity of each sample was expressed in terms of IC_{50}, and was calculated from the graph after plotting inhibition percentage against extract concentration. DPPH assay was carried out after making some modifications in the standard protocol (Hsu *et al.,* 2007). 3 ml of 0.1 mM DPPH solution was mixed with 1 ml of various concentrations (100 to 300 µg/ml) of leaf extract. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. The experiment was replicated in three independent assays. BHT was used as positive controls. Inhibition of DPPH free radical in percentage was calculated by the formula:

$$\text{Inhibition (\%)} = [(A_{control}-A_{test})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control (BHT) and A_{test} is the absorbance of reaction mixture samples (in the presence of sample). All tests were run in triplicates (n=3), and average values were calculated.

IC_{50} value

Inhibition Concentration (IC_{50}) parameter was used (Brand-Williams *et al.,* 1995) for the interpretation of the results from DPPH method. The discoloration of sample was plotted against the sample concentration in order to calculate the IC_{50} value. It is defined as the amount of sample necessary to decrease the absorbance of DPPH by 50 %.

Determination of $ABTS^+$ scavenging activity

For ABTS assay, the procedure followed was the method of Dimitrina (2010) and Roberta (1999) with some modifications. ABTS was dissolved in water to make a concentration of 7 mmol/L. $ABTS^+$ was produced by reacting the ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the test of samples, the $ABTS^+$ stock solution was diluted with 80% methanol to an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 4.85 ml of diluted $ABTS^+$ to 0.15 ml of stock samples solution (final concentration 1mg/ml), the absorbance reading was taken 6 min after the initial mixing. BHT (0.1 mg/ml) was used as positive controls. The activities of the samples were evaluated by comparison with a control (containing 4.85 ml of ABTS solution and 0.15 ml of 80% Methanol). Each sample was measured in triplicate and averaged. This activity is given as percentage $ABTS^+$ scavenging that is calculated by the following formula:

$$ABTS^+ \text{ scavenging activity (\%)} = Ac - As/Ac \times 100$$

Where, A_C is the absorbance value of the control and A_S is the absorbance value of the added samples test solution. The antioxidant capacity of test compounds was expressed as EC_{50}, the concentration necessary for 50% reduction of $ABTS^+$.

RESULTS

Statistical analysis: Results are expressed as the mean±SD for three replicates of each place by using SPSS version 16.

Proximate composition – Principal components, vitamin and carotenes

Moisture content of the leaves (Table 1) is 11% lower than the corresponding fruits. The data (Table 1) revealed a substantial higher percentage of principal nutrients in *T. dioica* leaves than the *T. dioica* fruits (protein +113.5 %, carbohydrate +102.72 %, lipid + 233.23 %, crude fiber +8.67%). *T. dioica* leaf evolved a richer source of the carotenoids as well as Vitamin C than its counterpart with the percentage gap of such stands at: Vitamin C +65.45% and carotenes +2664.05%. The principal components in general and the carotenoids in particular of the *T. dioica* leaf may place it at a higher position to act as a nutrient pool for the fight against Vitamin deficiency - Vitamin C and Vitamin A to be precise.

Table 1. Chemical constituents of *Trichosanthes dioica* leaves and fruits

Experimentation		Trichosanthes dioica leaves	Nutrient Composition of Trichosanthes dioica fruit (Gopalan *et al.,* 2004)	% Comparison W.R.T. Trichosanthes dioica fruits
	pH	7.28±.04	--	--
	Moisture (gm%)	81.68±0.48	92	-11.34
	Protein (gm%)	4.27±0.57	02	+113.5
	Carbohydrate (gm%)	4.46±0.28	2.2	+102.72
Proximate composition	Ash (gm%)	2.39±0.13	--	--
	Crude fibre (gm%)	3.26±0.11	3.0	+8.67
	Lipid (gm%)	1±0.05	0.3	+233.33
	Vitamin C (mg%)	47.98±2.85	29.0	+65.45
Vitamins	Carotene (µg%)	4229 ±11.22	153.0	+2664.05
	Calcium (mg%)	21.36±0.3	30.0	-28.80
	Iron (mg%)	102.41±0.46	1.7	+5924.12
Minerals	Potassium (mg%)	2.5±.05	83.0	-96.99
	Sodium (mg%)	0.08±.006	2.6	-96.92
Antinutrional factor	Phytate (mg%)	2.4±.073	08	-70.00
	Oxalate (mg%)	35.11±3.07	07	+401.57

Values are presented as mean ± SD, n = 3.

Table 2. Phytochemical content and DPPH free radical Antioxidant activity of *Trichosanthes dioica* leaves and fruits

Experimentation	*T. dioica* Leaves	*T. dioica* Fruits	% Comparison of *T. dioica* leaves W.R.T. T. *dioica* fruits	Standard BHT	% Comparison of *T. dioica* leaves W.R.T. Antioxidant activity of Standard BHT
Total polyphenol mg/gm dry extract	186.21 ± 8.78	15.94 (Alam *et al.,*2011)	+1068.19	--	--
Total flavonoids mg/gm dry extract	229.3± 5.65	29.98 (Alam *et al*., 2011)	+664.84	--	--
DPPH Free Radical scavenging assay IC_{50} µg/ml	170.78± 11.73	203.3 (Kavitha,2014)	-16.00	28.19±1.80	+505.81

Values are presented as mean ± SD, n = 3

Table 3. Total Hydrolysable Tannin content and $ABTS^+$ Free Radical scavenging activity of *Trichosanthes dioica leaf extract*

Sample	Total hydrolysable tannins	ABTS Radical scavenging assay IC_{50} µg/ml	% Comparison of *T. dioica* leaves W.R.T. Antioxidant activity of Standard BHT
Trichosanthes dioica leaf extract	14.88±1.22	140.65± 21.72	--
Standard BHT	--	75.37±0.045	-86.61

DISCUSSION

The present study encompasses the analytical evaluation of chemical constituents, phytochemical contents and antioxidant activities of *T. dioica* leaves and a comparative interpretation of the result w.r.t**.** the already known and established nutrient composition of *T. dioica* fruits.The nutrient contents of plant leaf were analyzed both on fresh weight and dry weight basis and expressed as gm/mg/µg per 100 gm sample (Moisture, carbohydrate, protein, vitamins were analysed on fresh weight basis and ash, crude fiber, lipid, minerals and antinutritional factors were analysed on dry weight basis as per standardised method). Tremendous work has been done on *T. dioica* fruit which is a well known daily vegetable with established medicinal values. The standard values of nutrients on the above fruit, given by ICMR, are available. The comparison of the experimental subject (leaves) with the ICMR values (of fruits) yielded the following outcome.

Minerals

From the experimentation (Table 1) it is found that although, *T. dioica* leaves are deficient in sodium, potassium and calcium contents (-96.92%, -96.99% and -28.80% respectively) in comparison to the fruits, but the iron content is remarkably higher (+5924.12%) than its counterpart (102.41±0.46 mg% and 1.7mg% respectively for leaf and fruit) (Table1), making the former a very efficient and economic source of iron

nutrition to the vulnerable section of the society fighting with malnutrition and anaemia.

Phytate and oxalate

Result and comparison with the ICMR values showed that the phytate content of *T. dioica* leaf (2.4±.073 and 8 mg respectively) is 70% lower that its fruit part where as the oxalate content (35.11±3.07 and 7mg respectively) is 401.57% higher than its counterpart. (Table1) The data points out to the fact that the higher oxalate content of the *T. dioica* leaves along with a lower level of calcium content make it unsuitable for consumption to the people suffering with Ca-deficiency diseases – as low Ca content may not supplement the Ca need of the consumer; the high oxalate content, on the other hand will act as a chelating agent to bind the available Ca within the system after metabolism. But, the same leaf may act as an important supplement to the people struck with Fe-deficiency disorders – as the higher level of Fe will supplement the Fe-need of the consumer to a great extent; the lower phytate content, in addition, will not act as a binder or chelating ligand to bind available Fe within the body after ingestion.

Phytochemical content

Result of present study of *T. dioica* leaf and established data of *T. dioica* fruit (Table 2) suggest that the *T. dioica* leaf portion is a better source of polyphenol (186.21 ± 8.78 mg/gm dry extract) and flavonoid (229.3± 5.65 mg/gm dry extract), being +1068.19 % and +664.84% higher than the corresponding contents in the fruit part.

DPPH free radical scavenging activity

IC_{50} value of *T. dioica* leaf portion (Table 2) (170.78± 11.73µg/ml) is 16.00% lower than the *T. dioica* fruit (203.3µ/ml), leading to the fact that leaf extract is more efficient quencher of DPPH free radicals than the fruit extract by as much as 16%. When compared to standard BHT regarding the DPPH free radical antioxidant activity, the leaf extract yielded better result than the fruit extract – being +505.81% and +621.18% less effective than std BHT to quench the radicals. That is, the leaf is 18.57% more effective as an antioxidant than the fruit – when DPPH free radical is concerned.

Total hydrolysable tannins and $ABTS^+$ free radical scavenging assay

A comparative analysis of *T. dioica* leaf and *T. dioica* fruit is not possible w.r.t. tannins and $ABTS^+$ free radical scavenging assay, as no data on these two experiments in previous studies on *T. dioica* fruit part is available. But, result of present study suggests that the leaf part is quite impressive in their capacity to scavenge $ABTS^+$ free radicals like DPPH free radicals. IC_{50} value of *T. dioica* leaf is remarkably good (140.65± 21.72µg/ml) when we compare it with the commercial standard antioxidant BHT (75.37±0.045µg/ml).

Conclusion

The principal components in general and the carotenoids in particular of the *T. dioica* leaf may place it at a higher position to act as a nutrient pool for the fight against Vitamin deficiency - Vitamin C and Vitamin A to be precise. *T. dioica* leaves are deficient in sodium, potassium and calcium contents in comparison to the fruits, but the iron content is remarkably higher (+5924.12%) than its counterpart making the former a very efficient and economic source of iron nutrition to the vulnerable section of the society fighting with malnutrition and anaemia. The higher oxalate content of the *T. dioica* leaves along with a lower level of calcium content make it unsuitable for consumption to the people suffering with Ca-deficiency diseases; the high oxalate content, on the other hand will act as a chelating agent to bind the available Ca within the system after metabolism.

But, the same leaf may act as an important supplement to the people struck with Fe-deficiency disorders – as the higher level of Fe will supplement the Fe- need of the consumer to a great extent; the lower phytate content, in addition, will not act as a binder or chelating ligand to bind available Fe within the body after ingestion. *T. dioica* leaf portion is a better source of polyphenols and flavonoids, being +1068.19 % and +664.84% higher than the corresponding contents in the fruit part. IC_{50} value of leaf extract shows that, it is a more efficient quencher of DPPH free radicals than the fruit extract by as much as 16%. When compared to standard BHT, the leaf extract yielded better result than the fruit extract – being 18.57% more effective as an antioxidant than the fruit. IC_{50} value of *T. dioica* leaf is remarkably good (140.65± 21.72µg/ml) when we compare it with the commercial standard antioxidant BHT (75.37±0.045µg/ml).

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