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THE BENEFICIAL EFFECTS OF TEMPERATURE ON ANTIOXIDANT AND ANTI-INFLAMMATORY POTENTIAL OF *Trichosanthes dioica* LEAF EXTRACT

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ABSTRACT

The medicinal plant provides remedies of many diseases due to their phytochemicals content. The objective of the present study is to focus on the stability of phytochemicals of *Trichosanthes dioica* leaf extract under wide range of temperature (100ºc, 150ºc 180ºc) applied for different time variation (10, 20, 30 min). The variation of phytochemicals (Polyphenol and Flavonoid), and *in vitro* antioxidant capacity (DPPH, ABTS, OH radical scavenging assay, Ferric reducing ability potential, total antioxidant capacity) and anti-inflammatory activity (inhibition of albumin denaturation assay) of the sample (methanolic leaf exract) with temperature are studied. Result suggests that the raw (without temperature effect) leaf extract of sample is good in phytochemicals content (182.87±1.51 mg/g polyphenol and 210.96±8.01mg/g flavonoid content) as well as free radical scavenging potential. Its potentiality in term of antioxidant activity increases with temperature and observed to be maximum at 180ºC (polyphenol content- 259.61^{g}±18.78 mg/g and flavonoid content-269.68^{h}±7.82mg/g) but less time period (10 to 20 min) is suggested to be optimum. So, It may be concluded that the phytochemicals content which are responsible for antioxidant potential, increases with temperature which suggests its uses as green leafy vegetables in daily dining as well as in pharmaceutical industry for drug discovery.

KEYWORDS: ***Polyphenol, Flavonoid, (2,2-diphenyl-1-picrylhydrazyl), Butyated Hydroxy toluene, Ferric reducing ability potential, Antioxidant.***

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INTRODUCTION

According to WHO, a medicinal plant is that which, in one or more of its organs, contains chemical substances usable for therapeutic purposes. These bioactive chemical substances of plants are often designated as phytochemicals, which protects them against microbial and pest infections.[1-4] *Trichosanthes,* a genus of family Cucurbitaceae is a perennial herb distributed in tropical Asia. *Trichosanthes dioica,* commonly khown as parwal*,* is used as tonic, febrifuge and in sub acute cases of enlargement of liver and spleen.[5] In Charaka Samhitha leaves and fruits used for treating alcoholism and jaundice. Leaves are used in odema and alopecia.[6]. Rai et *al.* showed the hypoglycaemic potential of an aqueous extract of *T. dioica* leaves.[7] Rai et *al* have reported that the LD_{50} o *Trichosanthes. dioica* is above 15g/kg.[8] Ghaisas et *al*. showed hepatoprotective activity of aqueous and ethanolic extract of *Trichosanthes dioica*[9]. Thorough investigation of nutritional and phytochemical property suggest that the leaf is remarkable source of iron, carotene, protein, polyphenol, flavonoids and more specifically galic acid, quercetin and catechin in raw leaf extract.[10-11] But *dioica* leaf may subject to various temperatures before consumption. Major plants fail to retain its potentiality in term of bioactive compounds through temperature stress. To create a general awareness about the beneficial effect of edible plant, it is necessary to analyse the stability of its bioactive compounds during domestic cooking at various temperatures. Despite, the various claims on *Trichosanthes dioica* Roxb medicinal uses, no attempt has been made to our best knowledge, to scientifically confirm the stability aspects or changes of that phytochemical over wide temperature range. So, the present concern of the study is to evaluate the thermal stability of phytochemicals of *trichosanthes dioica* leaves. In this study three different temperature (100ºc, 150ºc, 180ºc) which are relevant to domestic cooking are applied for three different time duration (10, 20, 30 min) on the samples to analyse the thermal stability of sample.

MATERIALS AND METHOD

Chemicals

2,2_-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,4,6-tri(2-pyridyl)- s-triazine (TPTZ), $FeCl_3$ $6H_2O$ [ferric chloride hexahydrate], 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, quercetin, gallic acid, catechin were purchased from Sigma Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). All other chemicals/solvent was supplied by E. Merck India.

Identification and authentication of sample

Whole plant of *Trichosanthes dioica* was submitted to the Herbarium of Calcutta University, Kolkata. It was indentified (Accession No. 20012) and authenticated by taxonomist of the Calcutta University Herbarium, Kolkata.

Sample Collection

The plant was collected from different district of West Bengal as well as from different local market.

Sample preparation

The leaves were shade dried for 3-4 days and grounded into powder and stored for further study. The leaf powder of samples were heated by placing them in hot air oven at 100ºc, 150ºc, 180ºc for 10, 20, 30min separately. Then 80% methanolic leaf extract were prepared from each heat treated powdered leaf as above and subjected to following in vitro antioxidant assay.

Determination of total polyphenol content

To measure the total polyphenol content, Folin–Ciocalteu assay was employed.[12] 0.2 ml of 80% methanolic extract of samples (1mg/ml) was added with 1 ml of Folin– Ciocalteu's phenol reagent (10 fold diluted). 0.8 ml of 2% Na_2CO_3 and 60% methanol were added successively. After 30 min incubation at room temperature the reaction mixtures were spectrophotometrically analysed at 740 nm. The calibration curve was plotted using gallic acid (20–100 µg/ml) as standard and the result of polyphenol content was represented as mg of gallic acid equivalent per g of dry extract.

Determination of flavonoid content

Aluminium chloride method was used to quantify total flavonoid content.[13] An aliquot of extracts (0.1ml) or the standard solution of quercetin (20 to 100 mg/ml) was 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 6th min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The absorbance was measured at 510 nm and the results were expressed as mg of quercetin equivalent per g of dry extract.

FRAP (Ferric reducing ability potential) Assay

FRAP values were evaluated by the method of Benzie and Strain.[14] To prepare working FRAP reagent, 50 ml of 300 mM acetate buffer (pH-3.6) was mixed with 5 ml of 40 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) dissolved in 40 mM HCl and 5 ml of 20 mM $FeCl_3$. 100 µg of extract was added to 3 ml of freshly prepared working FRAP reagent. The absorbance at 593 nm was measured immediately and after 4 min of incubation at 37 ºC. The change in absorbance was recorded as the final absorbance. For plotting calibration curve, $FeSO_4.7H_2O$ was used as standard at various concentrations (100-500 µM/l). The ferric reducing ability of sample was expressed as FRAP value (µM of Fe^{2+} equivalent).

Total antioxidant activity by the phosphomolybdenum method

The total antioxidant activities of the plant leaf extract were evaluated by the phosphomolybdenum complex formation method.[15] 100-300µg different concentration of each leaf extract were added to test tube containing 3 ml of distilled water and mixed with 1 ml of reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM ammonium molybdate).The vials were capped and incubated in water bath at 95°C for 90 minutes. After, cooling, the absorbance was measured at 695 nm against the reagent blank. The antioxidant activity is measured against an ascorbic acid calibration curve.

DPPH (2,2-diphenyl-1-picrylhydrazyl)radical scavenging activity [16]

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 against methanol blank. . The solution without any extract and with DPPH and methanol was used as control.

$$\text{DPPH radical scavenged (\%)} = [(A_{control} - A_{sample})/ A_{control}] \text{ X } 100.$$

$A_{control}$ = Absorbance of control, A_{sample} = Absorbance of sample

IC_{50} value of each sample was determined from the graph between sample concentration and the percentage of DPPH radical inhibition.

Determination of ABTS$^+$ [2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)] radical scavenging activity [17-18]

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 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. 4.85 ml of diluted ABTS+ was added to 0.15 ml of samples solution of different con. (100-300 µg), and the absorbance was taken 6 min after the initial mixing. BHT (0.1 mg/ml) was used as standard. 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).

$$\text{ABTS+ scavenging activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \text{ X } 100.$$

$A_{control}$ = Absorbance of control, A_{sample} = Absorbance of sample

The results were expressed as EC_{50}.

Hydroxyl (OH) radical scavenging activity [19]

Different concentrations (50, 100 and 150 µg) of sample extracts were taken separately in tubes. 1 ml of Iron EDTA (0.1% ferrous ammonium sulphate and 0.26% EDTA) was mixed with 0.5 ml of EDTA (0.018%) and 1 ml of DMSO (0.85% in 0.1 M phosphate buffer with pH 7.4). To commence the reaction 0.5 ml of ascorbic acid (0.22%) was added and held in a water bath at 80 – 90 ºC for 15 min. 1 ml of ice cold TCA was added to stop the reaction. 3ml of Nash reagent (75 g of ammonium acetate was mixed with 3 ml of glacial acetic acid and 2 ml of acetyl acetone and the volume was made up to 1 L with distilled water) was finally added and incubated for 15 min at room temperature for yellow colour development. Then the absorbance was taken at 412 nm against reagent blank.

$$\text{Hydroxyl radical scavenged (\%)} = [1 - (\text{sample absorbance} \setminus \text{control absorbance})] \text{ X } 100$$

The activity is expressed as EC_{50}.

***In vitro* lipid peroxidation inhibition assay** [20-21]

Freshly excised goat liver was processed to get 10% homogenate in cold phosphate buffer saline, pH- 7.4 using homogenizer and filtered to get clear homogenate. Different concentration (100-300 µg) of crude leaf extract was added to liver homogenate. Lipid peroxidation was initiated by adding 100µl of 15mM $FeSO_4$ solution to 3ml of tissue homogenate. After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated for 30 min in boiling water bath. The intensity of pink coloured complex formed was measured at 535 nm. A control was prepared without any sample.

$$\text{The percentage inhibition} = [(A_{control} - A_{sample})/ A_{control}] \text{ X } 100.$$

$A_{control}$ = Absorbance of control, A_{sample} = Absorbance of sample

The results were expressed as IC_{50} Value.

Inhibition of albumin denaturation[22]

The reaction mixture, consisting of test extract at different concentrations (100-300µg) and 1% aqueous solution of bovine albumin fraction were incubated at 37ºC for 20 min and then heated at 51ºC for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. Percent inhibition of protein denaturation was calculated as follows and IC_{50} value was calculated.

$$\text{Percentage inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \text{ X } 100$$

$A_{control}$ = Absorbance of control, A_{sample} = Absorbance of sample

STATISTICAL ANALYSIS

Statistical Analysis was performed with help of SPSS 17. Descriptive statistical analysis was performed to calculate the means with corresponding standard deviation (SD). Also One Way Analysis of variance (ANOVA) followed by post hoc Tukey's test was performed to compare the mean values. $p<0.05$ was taken to be statistically significant.

RESULT

Changes in phytochemicals upon cooking may result from two opposite phenomena: (1) thermal degradation, which reduces their concentration, and (2) a matrix softening effect, which increases the extractability of phytochemicals, resulting in a higher concentration with respect to the raw material. The final effect of cooking on phytochemical concentration depends on the processing parameters, the structure of food matrix, and the chemical nature of the specific compound.[23] So, it varies from sample to sample.

Table 1

Comparison of Yield percentages and phytochemical content at different high temperature and time.

Temp ºc	Time(min)	Yield percentages %gm	Total polyphenol content mg/g extract	Total flavonoid content mg/g extract
Raw		16.50^{a} ±.79	182.87^{a}±1.51	210.96^{de}±8.01
100	10	17.00^{ab} ±.74	201.50^{cd}±9.47	221.62^{ef}±7.17
100	20	15.58^{cd} ±1.16	209.60^{cd}±6.68	199.46^{c}±6.39
100	30	13.33^{h} ±.49	186.21^{ab}±8.79	170.88^{a}±5.87
150	10	14.67^{c} ±.49	220.57^{de}±7.97	228.45^{fg}±4.72
150	20	4.17^{i} ±.57	224.68^{ef}±7.72	200.23^{cd}±4.42
150	30	**18.42^{i} ±.67**	198.64^{bc}±11.90	186.71^{b}±4.17
180	10	9.33^{e} ±.88	225.54^{ef}±5.42	**269.68^{n}±7.82**
180	20	9.17^{eg} ±.94	227.63^{ef}±14.18	229.53^{fg}±9.47
180	30	9.50^{eg} ±.52	**259.61^{g}±18.78**	169.76^{a}±16.64

Values are represented as mean ± SD. Values followed by different superscript letter(s) (such as a,b,c etc) within each column denote that there are significant differences between the mean values at P < 0.05 level done by Tukey test.

Table 2

In vitro antioxidant assay at different temperature and time period

Temp ºc	Time (min)	FRAP mM Fe++ equivalent	TAC mg/g	DPPH IC_{50} value µg	ABTS EC_{50} value µg	OH IC_{50} value µg	LP IC_{50} value µg	AD IC_{50} value µg
Raw		841.60^{a}±77.13	387.84^{bc}±5.80	170.86^{efg}±11.82	140.64^{fgijk}±21.71	1104.84^{i}±117.13	327.39^{g}±23.52	181.86^{fgi}±1.69
100	10	901.31^{ab}±86.92	427.52^{cde}±33.70	166.83^{ef}±7.44	127.88^{efgij}±23.65	662.90^{e}±42.20	256.11^{de}±10.15	172.12^{efg}±3.42
100	20	979.99^{bc}±66.19	397.03^{bcd}±40.19	166.36^{ef}±11.01	123.45^{defgi}±22.95	311.98^{bc}±7.05	227.29^{cd}±25.79	109.33^{ab}±29.79
100	30	1323.93^{fg}±77.32	382.19^{bc}±40.76	163.38^{ef}±8.89	119.33 ^{cdefg}±19.60	334.24^{bc}±3.97	225.54^{cd}±42.44	158.79^{def}±11.00
150	10	1088.97^{d}±50.14	428.96^{cde}±32.95	158.29^{cde}±9.58	114.25^{bcdef}±18.85	351.77^{cd}±12.68	207.84^{bc}±6.30	147.56^{cde}±35.03
150	20	1199.92^{e}±32.96	370.23^{ab}±14.47	150.45^{bcd}±9.19	107.39^{abcde}±15.32	282.75^{b}±57.78	184.69^{ab}±30.85	142.26^{cd}±7.01
150	30	1237.14^{ef}±31.77	348.88^{ab}±23.24	144.21^{b}±6.78	102.60^{abcd}±11.23	343.88^{bc}±53.58	272.48^{ef}±52.98	166.73^{def}±24.30
180	10	964.21^{bc}±61.71	484.71^{i}±46.06	**126.37^{a}±7.69**	**80.47^{a}±9.00**	**175.44^{a}±0.87**	**164.90^{bc}±9.52**	**98.07^{a}±0.96**
180	20	**1621.86^{i}±73.44**	**511.96^{i}±42.45**	142.54^{b}±7.80	96.27^{ab}±11.81	217.64^{a}±5.90	177.65^{ab}±28.40	131.40^{bc}±23.40
180	30	1416.14^{h}±77.05	329.90^{a}±25.84	147.16^{bc}±8.07	100.41^{abc}±12.02	314.32^{bc}±1.18	215.12^{a}±32.93	145.47^{cd}±7.77
Standard µg /ml				BHT 28.19±1.8	BHT 75.01±0.50	Ascorbic acid 72.50±1.14	BHT 60.67±0.5	Aspirin 73.53±0.7

Values are represented as mean ± SD. Values followed by different superscript letter(s) within each column are significantly different at P < 0.05 by Tukey test.

FRAP- Ferric reducing ability potential, TAC- Total antioxidant capacity, DPPH-DPPH radical scavenging assay, ABTS- ABTS radical scavenging assay, OH- Hydroxyl radical scavenging assay, LP- Lipid peroxidation inhibition assay, AD- Albumin denaturation assay.

Table 3
Correlation matrix (Pearson's correlation coefficient) between various mean values of antioxidant assay of sample leaf extract

	POLY	FLAV	TAC	FRAP	DPPH	ABTS	AD	LP
FLAV	.084		.703**	-.083	.031	-.125	.133	-.031
TAC	.080	.703**		.046	-.119	-.149	.020	-.227*
FRAP	.104	-.083	.046		-.072	.005	-.020	-.013
DPPH	-.775**	.031	-.119	-.072		.740**	.460	.671**
ABTS	-.547**	-.125	-.149	.005	.740**		.379**	.571**
AD	-.591**	.133	.020	-.020	.460**	.379**		.690**
LP	.-642**	-.031	-.227*	-.013	.671**	.571**	.690**	
OH	-.523**	.141	-.021	-.183*	.509**	.479**	.567**	.715**

Correlation is significant at the 0.05 level. ** Correlation is significant at the 0.01 level.
FRAP- Ferric reducing ability potential, TAC- Total antioxidant capacity, DPPH-DPPH radical scavenging assay, ABTS- ABTS radical scavenging assay, OH- Hydroxyl radical scavenging assay, LP- Lipid peroxidation inhibition assay, AD- Albumin denaturation assay.

DISCUSSION

Result shows the yield percentages of sample vary with temperature and the highest value was observed at 150ºC (30min). Table 1 suggests an impressive value of polyphenol (182.87±1.51mg/g) in raw sample leaves. But by breaking the usual behaviour of polyphenol to high temperature, here it is observed that the rise of temperature applied for increasing time duration showed a sharp upward trend. Individually 20 min time duration for 100 ºc, and 150 ºc and 30 min for 180 ºc is suggested to be best for polyphenol extraction. But overall, high temperature at 180ºc and long time duration (30min) are optimum for polyphenol extraction (259.61±18.78mg/g**)**. The polyphenolic composition of food is highly variable both qualitatively and quantitatively.[23-24] In plants, phenolic compounds occur in free forms as well as covalently bound with macromolecules or packed in cellular organs or cell wall components.[25-26] Cooking may cause complex physical and chemical changes in phenolic compounds, including release from bound forms, degradation, polymerisation and oxidation.[27-28] So there may be some structural modification, (at high temperature) that may result in increased polyphenol release. Similar observation of positive role of temperature on phenolic content has also been reported by Naeem et al.[29] It is observed that flavonoid release is best at 10 min time duration for each set of temperature. But as a whole it is observed that high temperature (180ºc) and lower time period (10min) resulted in better flavonoids (269.68±7.82) release. In the study of Sharma et al it was showed that heating had a positive effect on all four flavonoid of onions.[30] The reason may be because of the conversion or release of glucosides or bound form into free derivatives. In most fruits and vegetables, flavonoids contain C-glycoside bonds and exist as dimers and oligomers, and heating or boiling results in the formation of monomers by the hydrolysis of C-glycosides bonds.[30] It was reported that regardless of the cooking treatment, quercetin- are relatively heat-stable.[31] So, thermal stability and hydrolysis of glycosides bond result in increase flavonoid content over domestic cooking temperature upto 180ºc. It is supported by previous study where Sathishkumar et al., showed that at higher temperatures, flavonoids diffused more quickly from the cell to the extracting solvent.[32] Table 2 suggest that three different time duration were applied for each set of temperature. 30 min time duration is best in term of DPPH, ABTS radical scavenging activity, lipid peroxidation and FRAP potential where as at 180 ºC, 10 min time duration is suggested to be best. But overall maximum radical scavenging potential is observed at 180ºC for 10 min time duration. Table 3 suggest that there is good correlation between phytochemicals (polyphenol, flavonoids) and *in vitro* antioxidant potential in term of IC_{50} values (DPPH, ABTS, FRAP, OH radical scavenging assay, Lipid peroxidation, Albumin denaturation). It is supported by a previous study where Singh et al., showed good correlation between flavonoid and DPPH radical scavenging potential, which is measure of in vitro antioxidant activity.[33] Increase in free phenolics and flavonoids content at 180ºC results in increase of free radical scavenging potential (DPPH, ABTS, OH), reducing capability (FRAP, Total antioxidant capacity), inhibition of lipid peroxidation and albumin denaturation potential of sample. The raw leaf extract is able to scavenge DPPH, ABTS, OH, and lipid peroxide free radicals in comparison to BHT and ascorbic acid standard. The results expressed in IC_{50} value (Lower indicates better). Denaturation of proteins is a well documented cause of inflammation.[20] So, albumin denaturation inhibition assay is a marker of anti-inflammatory assay. In comparison to IC_{50} value of Aspirin, (a standard anti-inflammation drug), the sample has anti- inflammatory potential which increases with temperature. Result (Table 2) depicted that the bioactive compounds of sample are not only thermally stable but also show greater performance up to 180 ºC.

CONCLUSION

Most of the green leafy vegetables are heated to different extent before consumption. So, the variation of phytochemical contents with heat treatment in comparison to raw condition is necessary to focus. The present study reveals that the phenolic compounds become more available with heat treatment. Temperature has significant effect on phytochemical profile quantitavely and antioxidant potential of sample. Only simple domestic cooking temperature brings about increase in disease preventing capacity of sample. So, this plant is good in nutritional, phytochemical, economic and even stability point of view. The plant with such combination can be used as supplement in many food preparations as nuetraceuticals at low cost. Since the phytochemicals of this sample are not heat sensitive, further extensive work on them may lead to a horizon of modern drug design and development.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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