IN VITRO ANTIOXIDANT ACTIVITIES OF *TETRASTIGMA THOMSONIANUM* PLANCH. LEAVES AND STEMS

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Summary

Tetrastigma thomsonianum is a common vegetable consumed in North East India. The study evaluated the antioxidant potential of leaves and stems of the plant using in vitro antioxidant assays. In order to find antioxidant properties, methanolic extracts were studied for TPC, TFC, ANT, RP, DPPH$^{\bullet}$, OH$^{\bullet}$, H_2O_2, NO$^{\bullet}$ radical scavenging activities at various concentrations. Leaves and stems were found to contain a noticeable amount of total phenolics and flavonoids. The antioxidant properties of the extracts may be due to higher phenolic and flavonoid content. The study suggested that *T. thomsonianum* can be used as a readily accessible source of natural antioxidants and as a possible food supplement.

Keywords: *Tetrastigma thomsonianum*, In vitro antioxidant activities, Phenolic content, Flavonoid content

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Introduction

The ethnomedical literature contains a large number of plants with potential antioxidant properties that can be used against diseases, in which reactive oxygen species (ROS) are thought to play a major role. An important source of antioxidants is the diet, which contains numerous plants with antioxidant activity, including the vegetables, spices and condiments (1, 2). A sufficient supply of antioxidants from the diet might help to prevent or delay the occurrence of pathological changes associated with oxidative stress. When diet fails to meet the antioxidant requirements, dietary supplement might be used to enhance health. Plants contain a number of phytoconstituents e.g. flavonoids, phenolics, coumarins, xanthenes, vitamin C, tocopherols etc. with ability to scavenge free radicals produced in our body.
Tetrastigma thomsonianum Planch. (Family: Vitaceae) is a herbaceous climber with dark-coloured usually flattened stem and found in North East India. Tender shoots and leaves are eaten which are acidic; It is very tasty when it is cooked with fish. It is commonly used as vegetable and known to have medicinal properties (3).

In the quest of finding out the extent of anti-oxidant activity of commonly consumed vegetable, *T. thomsonianum*, of North East India, the present study has been aimed to analyze the same. This is the first attempt on searching the antioxidant activity in leaves and stems of *T. thomsonianum* which is used as a vegetable by people of Assam, Manipur and Meghalaya in India.

Materials and methods

Chemicals

L-Ascorbic acid, Quercetin, Butylated hydroxyl toluene (BHT), β-carotne, Curcumin Crystalline, 2-Deoxy-D-ribose, Ferric chloride anhydrous, 2-Thiobarbituric acid, Linoleic acid, Tween 40, Potassium ferricyanide, Sulphanilic acid, N-(1-Naphthyl) ethylene diamine dihydrochloride, Sodium hydroxide pellets, Sodium carbonate anhydrous, Sodium chloride were purchased from HiMedia Laboratories Pvt. Limited (Mumbai, India). Disodium hydrogen phosphate and gallic acid were purchased from LOBA-Chemie Indoaustranal Co. (Mumbai, India). 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethylenediaminetetraacetic acid disodium salt, Trichloroacetic acid and Potassium dihydrogen phosphate were purchased from RANKEM Pvt. Ltd. (Mumbai, India). All the chemicals listed above and others used in the experiment were of analytical grade.

Plant material

The leaves and stems of *Tetrastigma thomsonianum* Planch. were collected from the plant in the month of November, 2009 from Dibrugarh, Assam. The plant was identified and a voucher specimen DU/LS/215 was deposited in the herbarium of the Department of Life Sciences, Dibrugarh University, Assam, India for future reference.

Preparation of the extracts

Shade dried leaves and stems of *Tetrastigma thomsonianum* were crushed in a mechanical grinder to reduce its size. The powder (500 g) was then extracted with 2.5 Lit. of petroleum ether (40-60ºC) in a Soxhlet apparatus until the powder became exhausted totally. The defatted material was then extracted with 2.5 Lit. of methanol in a Soxhlet apparatus until the powder became exhausted totally. Resulting methanolic extracts were filtered, concentrated, dried and were stored in a desiccator for use in subsequent experiments.

Evaluation of antioxidant activity

Total phenolic content

The total concentration of phenolics in methanolic extract of *T. thomsonianum* leaves and stems were determined according to the the methods given in the literature (4, 5) involving Folin–Ciocalteu reagent and gallic acid as standard. 1 ml of extract solution containing 2000 µg extract was added to a volumetric flask. 45 ml distilled water and 1 ml Folin–Ciocalteu reagent was added and flask was shaken vigorously. After 3 min, a 3 ml of Na_2CO_3 (2%) solution was added and the mixture was allowed to stand for 2 h by intermittent shaking. Absorbance was measured at 760 nm. The phenolic compound content (TPC) was determined as Gallic acid equivalents using the following linear equation based on the calibration curve: $A = 0.004C + 0.007$, $R^2 = 0.996$. A is the absorbance, and C is Gallic acid equivalents (GAE) (µg). The test was performed in triplicate and average value was represented.

Total flavonoid content

Total soluble flavonoid content of the extracts was determined with aluminium nitrate using quercetin as the standard. Total flavonoid content (TFC) was determined using the Dowd method as adapted by Arvouet-Grand et al. (1994) (6). Briefly, 1 ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the methanolic extracts (2000 mg ml^{-1}). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin graph: Absorbance, $A = 0.021$ C - 0.003, $R^2 = 0.996$. C is the quercetin equivalents (QE) (µg). The test was performed in triplicate and average value was represented.

β-Carotene bleaching assay

In this assay antioxidant capacity (ANT) is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (7). A stock solution of β-carotene– linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade). 25 µl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml of oxygenated distilled water was added with vigorous shaking; 2.5 ml of this reaction mixture was dispersed to test tubes and 0.5 ml of various concentrations (0.5–4.5 mg ml^{-1}) of the extracts in methanol were added and the emulsion system was incubated for up to 2 h at 50ºC. The same procedure was repeated with the positive control BHT and a blank. After this incubation period, absorbance of the mixtures was measured at 490 nm. Measurement of absorbance was continued until the color of β-carotene disappeared. Antioxidative activities of the extracts were compared with those of BHT at 0.5 mg ml^{-1} and blank consisting of only 0.5 ml methanol.

The rate of β-carotene bleaching for the extracts and BHT was calculated according to first-order kinetics, as described in Al-Saikhan et al. (1995) (8):

$$R = \ln \frac{1}{t} \times \frac{A_{t=0}}{A_{t=t}}$$

where t is the time in minutes, $A_{t=0}$ is the initial absorbance of the emulsion immediately after sample preparation (t = 0 min) and $A_{t=t}$ is the absorbance recorded every 15 min after the beginning of the experiment. The average rate of β-carotene degradation (R) was calculated according to the first-order kinetics Al-Saikhan et al. (1995) (8).

The percent of antioxidant activity (ANT) was calculated using the equation:

$$\% \quad ANT \quad = \quad \frac{(R_{\text{Control}} - R_{\text{test}})}{R_{\text{Control}}} \times 100$$

where R_{Control} and R_{Sample} are average bleaching rates of the negative control and the antioxidant (plant extracts or BHT), respectively.

Reducing power

The reducing power (RP) of methanolic extracts of *T. thomsonianum* leaves and stems were determined according to the method of Oyaizu (1986) (9). 1ml of extract of different concentrations (50, 100, 150, 200, 250 and 300 µg/ml) in distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml, 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. 2.5 ml of trichloroacetic acid (10%) was added to this mixture, which was then centrifuged at 3000 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml, 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. All the tests were performed in triplicate. BHT was used as a reference standard. Gradual enhancement of absorbance with concentration indicates the reducing power of sample.

DPPH radical (DPPH$^{\bullet}$) scavenging activity

Radical scavenging activity of plant extracts against stable DPPH (1, 1-diphenyl-2-picrylhydrazyl hydrate was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep—violet to light—yellow) were measured at 517 nm on a UV/Visible spectrophotometer (Shimadzu UV-1700 PharmaSpec, Kyoto, Japan). The free radical scavenging activity of methanolic extracts of *T. thomsonianum* leaves and stems were measured by DPPH (1, 1-diphenyl-2-picryl-hydrazyl) employing the method described by Blois, (1958) (10) with slight modification. 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 1 ml of various concentrations (5-80µg/ml) of methanolic extracts and volume was made up to 3 ml with methanol. After 30 min, absorbance was measured at 517 nm. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. All the tests were performed in triplicate. Ascorbic acid was used as a reference compound.

The capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ DPPH}^{\bullet} \text{ scavenging activity} = \frac{(A_{\text{Control}} - A_{\text{test}})}{A_{\text{Control}}} \times 100$$

Where A_{Control} is the absorbance of the control reaction and A_{test} is the absorbance in the 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 (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%.

Hydroxyl radical ($OH^{\bullet}$) scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and methanolic extracts of *T. thomsonianum* leaves and stems for hydroxyl radical generated by Fe^{3+}-Ascorbate–EDTA–H_2O_2 system (Fenton reaction) according to the method of Kunchandy *et al.* (1990) (11). The reaction mixture contained in a final volume of 1.0 ml, 100 µl of 28 mM 2-deoxy-D-ribose in 20 mM KH_2PO_4-KOH buffer of pH 7.4, 500 µl of the selected concentrations of extract (25, 50, 100, 150, 200, 250 and 300 µg/ml) in KH_2PO_4-KOH buffer (20 mM, pH 7.4), 100 µl of 1.04 mM EDTA, 100 µl 200 mM $FeCl_3$, 100 µl of 1.0 mM H_2O_2 and 100 µl of 1.0 mM ascorbic acid was incubated at 37 ºC for 1 h. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8 %) were added to the test tubes and were incubated at 100 ºC for 20 min. After cooling, absorbance was measured at 532nm against control containing deoxyribose and buffer. BHT was used as a positive control. Reactions were carried out in triplicate. The percentage inhibition was determined by comparing the results of the test and control compounds. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation and is calculated as:

$$\% \text{ OH}^{\bullet} \text{ radical Scavenging activity} = \frac{(A_{\text{Control}} - A_{\text{test}})}{A_{\text{Control}}} \times 100$$

Where A_{Control} is the absorbance of the control reaction and A_{test} is the absorbance in the 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 µg/ml) of extract that inhibits the formation of OH^{-} radicals by 50%.

Hydrogen peroxide (H_2O_2) radical scavenging activity

The ability of extracts to scavenge H_2O_2 was determined according to the method of Ruch *et al.* (1989) (12). A 40 mM solution of H_2O_2 was prepared in phosphate buffer (pH 7.4). Extracts of selected concentration (50, 100, 150, 200, 250 and 300 µg/ml) in distilled water were added to a H_2O_2 solution (0.6 ml, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without H_2O_2. Ascorbic acid was used as a standard antioxidant. The percentage of H_2O_2 scavenging of both the extracts and standard compounds was calculated.

The percentage inhibition was calculated as:

$$\% \ H_2O_2 \text{ radical Scavenging activity} = \frac{(A_{Control} - A_{test})}{A_{Control}} \times 100$$

Where $A_{Control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts The antioxidant activity of the extracts were expressed as IC_{50}.

Nitric oxide ($NO^{\bullet}$) radical scavenging activity

Nitic oxide radical scavenging activity of the extracts was determined according to the method of Vaijanathappa et al., 2008 (13). The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4ml), Phosphate buffer saline (PBS) (1 ml) and the extract or standard solution (1 ml) of various concentration (10,20,40,100,150,200 µg/ml) was incubated at 25ºC for 150 min. After incubation, 0.5 ml of the reaction mixture was removed, 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of the diazotization reaction, 1 ml of N-(1-Naphthyl) ethylene diamine dihydrochloride was added, and the mixture was allowed to stand for 30 min in diffused light. The absorbance was measured at 540 nm. Curcumin was used as a standard antioxidant.

The percentage inhibition was calculated as:

$$\% \ NO^{\bullet} \text{ radical Scavenging activity} = \frac{(A_{Control} - A_{test})}{A_{Control}} \times 100$$

Where $A_{Control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts The antioxidant activity of the extract were expressed as IC_{50}.

Statistical analysis

All the experiments are carried out in triplicates. The experimental results are expressed as mean ± SEM (standard error of mean) of triplicate measurements and the results were processed using Microsoft Excel.

Results and discussion

Estimation of total phenolic:

Total phenolic contents compounds of methanolic extracts of *T. thomsonianum* leaves and stems were expressed as µg of gallic acid equivalent (GAE) per mg of dry weight of extract. The level of total polyphenolic compounds were 111.88±0.17 µg and 61.25±0.09 µg of GAE per mg of methanolic extracts of leaf and stem respectively (Table 1.).

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant metabolites because of their radical scavenging ability due to their hydroxyl groups.The phenolic compounds may contribute directly to antioxidant action. The presence of phenolic of this extract may be responsible for the antioxidant activity of methanolic extract of *T. thomsonianum* leaves and stems.

Estimation of Total Flavonoids:

Total flavonoids content of methanolic extracts of *T. thomsonianum* leaves and stems were found to be 0.72±0.02 µg and 0.97±0.06 µg of quercetin equivalent per mg of methanolic extracts of leaf and stem respectively (Table 1.).

It has been recognized that flavonoids exert considerable antioxidant activity and exhibit substantial beneficial effects on human nutrition and health. The mechanisms of action of flavonoids are thought through scavenging or chelating process (14, 15). The extracts contain substantial quantity of flavonoids which may contribute in considerable function for the antioxidant activity of methanolic extracts of *T. thomsonianum* leaves and stems.

Total antioxidant activity by β-carotene–linoleic acid method:

Using the β-carotene/linoleic acid method, methanolic extracts of *T. thomsonianum* leaves and stems showed different patterns of antioxidant activities. The most active extract was of leaf at 3.0 mg ml^{-1} concentration (93.89 ± 0.64%). This activity was followed by extract of stem at 3.0 mg ml^{-1} concentration (89.68 ± 0.49%). BHT showed total antioxidant activity at 0.5 mg ml^{-1} of 95.69±0.68%(Table 1.). Figure 1. shows total antioxidant activity of methanolic extracts of leaf and stem.

Heat-induced oxidation of an aqueous emulsion of β-carotene and linoleic acid was employed as a test for measuring total antioxidant activity of the extracts. In this particular model, linoleic acid free radical, whose formation is heat-induced, reacts with β-carotene, which undergoes rapid discoloration. The presence of an antioxidant can reduce the extent of β-carotene destruction by reacting with the linoleate free radical or any other free radical formed within the system (16, 17). Antioxidant activity was measured as a percentage inhibition of lipid peroxidation (Table 1). All the extracts were able to reduce the rate of degradation of β-carotene in comparison with the control. The extract of leaf was stronger antioxidant than the corresponding extract of stem and their activity did not differ from the activity of BHT.

Reducing power determination:

The reducing power of methanolic extract of *T. thomsonianum* leaves and stems is indicated in Figure 2. The reducing power was measured in term of absorbance at 700 nm. The increasing values of absorbance with increasing amount of sample recommend the reducing power of test extract and standard BHT.

For the measurements of the reductive ability, the Fe^{3+} - Fe^{2+} transformation has been investigated. The reducing capacity of an extract may serve as a significant indicator of its potential antioxidant activity (18). The antioxidant activity has been reported to be concomitant with development of reducing power (19).The reducing power of methanolic extracts of *T. thomsonianum* leaves and stems were increased with increasing amount of sample.

Inhibition of DPPH radical:

The reduction capacity of DPPH radicals was measured at 517nm. Figure 3. illustrates a steady increase of percentage scavenging activity with concentration of methanolic extract of *T. thomsonianum* leaves, stems and reference standard ascorbic acid. The IC_{50} values were found to be 21±0.67 (µg/ml), 61±1.59 (µg/ml) and 12±0.27 (µg/ml) for extracts of leaf, stem and ascorbic acid respectively (Table 1.).

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (20). The reduction capability of DPPH radicals was determined from the decreasing its absorbance at 517 nm with the increasing concentration, which is induced by antioxidants present in the extracts.

Inhibition of hydroxyl radical:

The hydroxyl radical scavenging activity is apparent from the raise of percentage scavenging activity with concentration of methanolic extracts of *T. thomsonianum* leaves, stems and reference standard BHT (Figure 4.). The IC_{50} values were found to be 140±2.32 (µg/ml), 110±1.78 (µg/ml) and 74±0.84 (µg/ml) for extracts of leaf, stem and BHT respectively (Table 1.).

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins (21). The inhibition of free radical-mediated deoxyribose damage was assessed by means of the Iron (II)-dependent DNA damage assay. The Fenton reaction generates hydroxyl radicals (OH^-) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. The extracts inhibited the Iron (II)-dependent deoxyribose damage in all concentrations.

Inhibition of hydrogen peroxide radical:

The results of hydrogen peroxide radical scavenging activity are shown in Figure 5. The methanolic extracts of *T. thomsonianum* leaves and stems scavenged hydrogen peroxide radical in a concentration dependant manner, which is clear from the gradual rise of graph with concentration. The result is comparable with that of reference standard ascorbic acid. The IC_{50} values were found to be 102±1.42 (µg/ml), 90±0.62 (µg/ml), and 70±0.27 (µg/ml) for extracts of leaf, stem and ascorbic acid respectively (Table 1.).

Hydrogen peroxide itself is not only very reactive, but it can sometimes be toxic to cell as it provides hydroxyl radical in the cells (22). Thus, removing H_2O_2 as well as O_2^- is very important for protection of food systems. The extracts exhibited H_2O_2 scavenging ability.

Inhibition of nitric oxide radical:

The methanolic extracts of *T. thomsonianum* leaves and stems inhibited nitric oxide radical in a concentration dependant manner and the result is comparable with that of standard curcumin (Figure 6.). The concentration of extracts of leaf and stem needed for 50% inhibition were found to be 126±1.63 (µg/ml), 84±1.37 (µg/ml) respectively, whereas 32±0.34 (µg/ml) for curcumin (Table 1.).

Nitric oxide radical (NO^-) generated from sodium nitroprusside at physiological pH was found to be inhibited by the extracts.

Table 1.: Total phenolic content (TPC), total flavanoid content (TFC), Total antioxidant activity (ANT), IC_{50} values of $DPPH^•$ radical, Hydroxyl radical ($OH^•$), hydrogen peroxide radical (H_2O_2), nitric oxide radical ($NO^•$) scavenging activity of methanolic (MeOH) extracts of leavess and stems of *Tetrastigma thomsonianum* Planch.

MeOH Extract	TPC (µg/mg)	TFC (µg/mg)	ANT (%)	IC_{50} (µg/ml)			
				DPPH	$OH^•$	H_2O_2	$NO^•$
Leaf	111.88±0.17	0.72 ±0.02	93.89 ± 0.64	21±0.67	140±2.32	102±1.42	126±1.63
Stem	61.25±0.09	0.97 ±0.06	89.68 ± 0.49	61±1.59	110±1.78	90±0.62	84±1.37
Std.	-	-	95.69±0.68 (BHT)	12±0.27 (Ascorbic acid)	74±0.84 (BHT)	70±0.2 (Ascorbic acid)	32±0.34 (Curcumin)

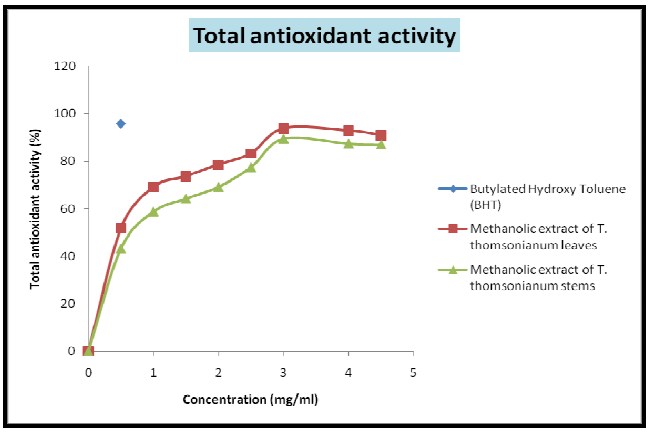

Figure 1. Total antioxidant activity by β-carotene–linoleic acid method.

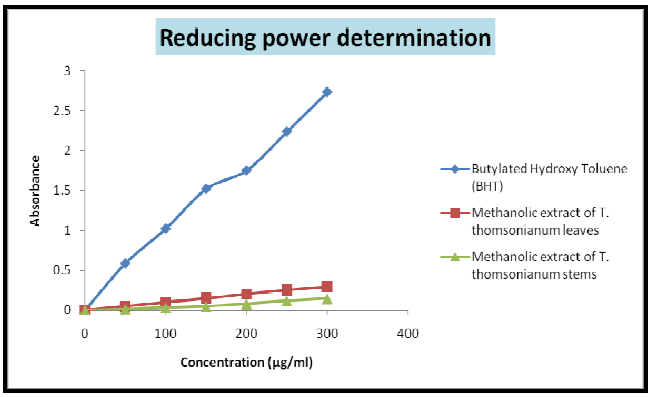

Figure 2. Determination of reducing power.

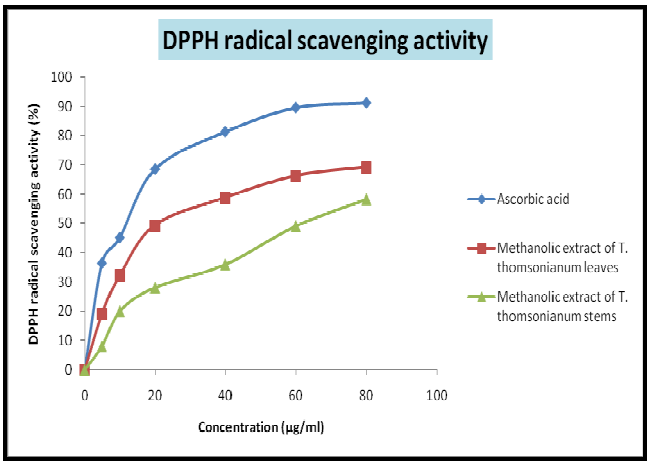

Figure 3. DPPH (1, 1-diphenyl -2-picryl-hydrazyl) radical scavenging activity.

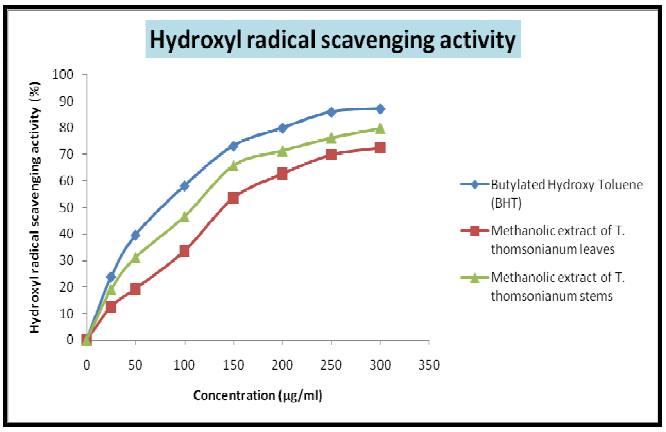

Figure 4. Hydroxyl radical scavenging activity.

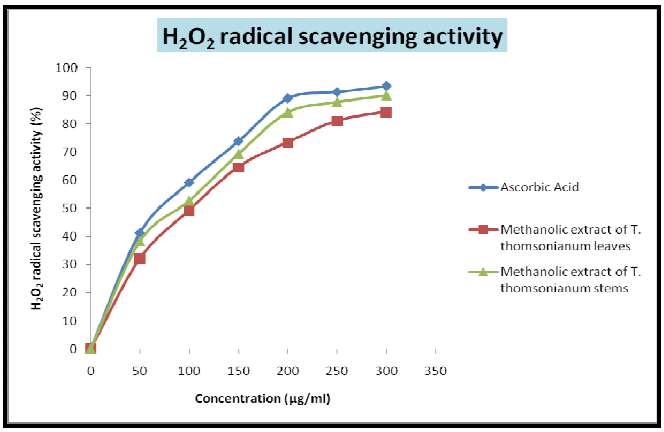

Figure 5. Hydrogen peroxide radical scavenging activity.

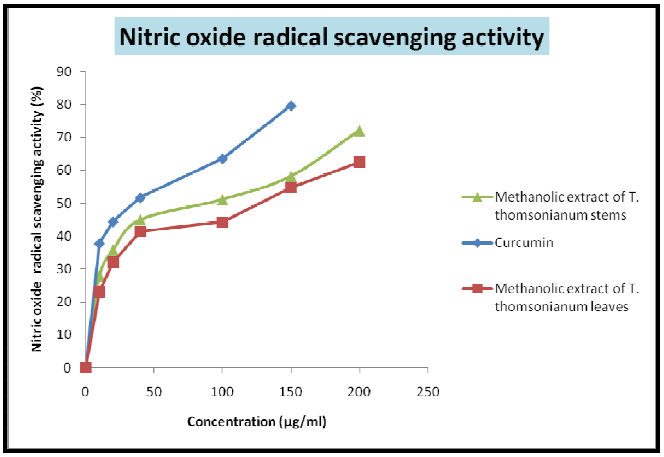

Figure 6. Nitric oxide radical scavenging activity.

Conclusion

The leaves and stems exhibited potent antioxidant activity by β-carotene bleaching activity, reducing power activity, DPPH$^{\bullet}$ radical, hydroxyl radical (OH$^{\bullet}$), nitric oxide radical (NO$^{\bullet}$) and hydrogen peroxide radical scavenging activities. In addition, the extracts were found to contain a higher amount of total phenols and flavonoids, which play a major role as antioxidants. As it is a vegetable, it can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

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