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In vitro Antioxidant and Radical Scavenging Capacities of Extracts from *Tinospora cordifolia*

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The aim of this investigation is to study the *in vitro* antioxidant and radical scavenging activity of *Tinospora cordifolia* (Willd.) extracts. The antioxidant components were initially extracted in methanol and subjected to fractionation in solvents of different polarity. The chloroform soluble fraction was then subjected to column chromatography using *n*-hexane with a gradient of chloroform upto 100 %, followed by methanol. Antioxidant potential and radical scavenging activity of the 12 fractions obtained from the column were investigated employing different antioxidant assays such as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation scavenging, ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazil (DPPH) scavenging, total phenolic content (TPC) and total antioxidant activity determination using ferric thiocyanate method. Using $ABTS^{\bullet+}$ decolourization assay and FRAP assay, *T. cordifolia* extracts showed a wide range of antioxidant activity. Trolox equivalent antioxidant capacity (TEAC) and FRAP values for various fractions ranged from 0.003-7.654 mM of trolox equivalents and 126.88-1516.80 mg/L of $FeSO_4$ equivalents, respectively. Using total phenolic content assay the amount of total phenolics for different fractions of *T. cordifolia* ranged from 4.84 ± 0.21-107.27 ± 1.37 mg/L. No significant co-relation was found between TPCs and TEAC (r^2 = 0.652) and FRAP assay (r^2 = 0.686). The EC_{50} values determined by using DPPH radical scavenging assay ranged from 83.26-588.31 µg of dried weight (DW)/mL of DPPH solution. Employing inhibition of lipid peroxidation assay by ferric thiocyanate method, the extracts showed inhibition of lipid peroxidation comparable to trolox. On the basis of the results obtained here, *T. cordifolia* may be considered as a rich source of antioxidants.

Key Words: *Tinospora cordifolia*, 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulpohonic acid), Radical cation, Trolox equivalent antioxidant capacity, Ferric reducing antioxidant power.

INTRODUCTION

In living systems, free radicals are generated as part of the body's normal metabolic process and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthine oxidase activity,

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atmospheric pollutants and from transition metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage. Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders. For instance, in diabetes, increased oxidative stress which co-exists with reduction in the antioxidant status has been postulated[1]. Currently, the possible toxicity of synthetic antioxidants has been criticized. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea and herbs may contribute to shift the balance toward an adequate antioxidant status. Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years[2].

Tinospora cordifolia (Willd.) Miers ex Hook. F. and Thoms is commonly known as Guduchi in Indian Sub-Continent and belongs to family Menispermaceae. It is a large, glabrous, deciduous climbing shrub. A variety of chemical constituents have been isolated from this plant and they belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides[3]. Different parts of this plant have been used for different ailments. The stem is used in dyspepsia, fevers and urinary diseases. The bitter principles which have been extracted and identified as columbin, chasmanthin and palmarin show antiperiodic, antispasmodic, antiinflammatory and antipyretic properties[4-6]. Studies on induced oedema and arthritis and on human arthritis have proved the antiinflammatory, antipyretic and hypoglycaemic activities of the water extract of this plant[7-9]. Its methanolic extract has also been shown to possess significant antifungal activity against *Dreschlera turcica*[10]. Besides pure extracts, *T. cordifolia* has also been used in the form of ayurvedic herbomineral formulation for antihyperglycaemic, antiulcer, hepatocurative, antiatherogenic, superoxide dismutase and antioxidative activity in rats[11-15].

Although some preliminary studies on antioxidative potential of the plant extracts and its herbomineral formulations have been carried out[14,16], but no comprehensive study on the radical scavenging and antioxidant capacity has been undertaken as yet. The objective of the present study is to evaluate radical scavenging and antioxidant potential of different organic extracts of *T. cordifolia* (Willd.) by using trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing antioxidant power (FRAP) assay, total phenolic contents (TPC) assay, DPPH free radical scavenging assay and total antioxidant activity using ferric thiocyanate.

EXPERIMENTAL

Tinospora cordifolia (Willd.) was purchased from a local market, Paparr Mandi, Lahore, in March 2008 and identified by Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore.

Extraction of antioxidant components: The shade-dried ground whole plant (10 Kg) was exhaustively extracted with methanol at room temperature. The extract

was concentrated to yield the solid residue (304 g). The whole residue was dissolved in distilled water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol successively. The chloroform-soluble fraction (240 g) was subjected to column chromatography over a silica gel eluting with *n*-hexane and gradient of chloroform up to 100 %, followed by methanol. Twelve fractions (Fr. 1-12) collected were investigated for total antioxidant capacity (TAC) using different assays.

Chemicals and standards: Standard antioxidants such as trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), butylated hydroxy anisole (BHA), gallic acid, ascorbic acid, quercetin and iron(II) sulphate, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, Follin-Ciocalteau's reagent and potassium persulfate (di-potassium peroxdisulfate) were purchased from Fluka (UK). HPLC grade ethanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, Scotland). Spectrophotometric measurements were made on UV-1700 PharmaSpec. UV-visible spectrophotometer, Shimadzu, Japan equipped with temperature control device. All the solutions were made in triplicate and experiments were performed three times. The results obtained were averaged.

$ABTS^{\bullet+}$ radical cation decolourization assay: ABTS radical scavenging assay is based upon the scavenging of ABTS radical cation generated from a reaction between ABTS and potassium persulfate. The antioxidant components reduce bluish green ABTS radical to colourless native ABTS depending upon their nature and amount on a time-dependant scale. Trolox is commonly used as standard antioxidant for comparison purposes. For the determination of trolox equivalent antioxidant capacity (TEAC) of the extracts, $ABTS^{\bullet+}$ decolourization assay protocol was followed[17]. ABTS radical cation was produced by a reaction between ABTS and potassium persulfate (7 mM and 2.45 mM final concentrations, respectively) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. To study the antioxidant activity of standard antioxidant and plant extracts, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 ºC. After addition of 10 µL of sample or standard antioxidant to 2.99 mL of diluted $ABTS^{\bullet+}$ solution (A = 0.700 ± 0.020), the absorbance was noted at 30 °C, with exactly 1 min intervals for 6 min. Appropriate solvent blanks were run in each assay for accurate readings. All determinations were carried out at least three times in succession and in triplicate at each separate concentration level of the standards. The percentage inhibition of absorbance was calculated by the following formula.

$$\text{Inhibition}_{(\text{at } 734 \text{ nm})}\ (\%) = (1 - I_f/I_o) \times 100$$

where I_o is the absorbance of radical cation solution before addition of sample/standard antioxidants and I_f is the absorbance after addition of the sample/standard antioxidants. The resultant data was plotted between concentration of antioxidants and that of trolox for the standard reference curve.

Total phenolic contents assay: Total phenolic contents of the extracts were determined by an reported method[18]. Stock solution of gallic acid was made by dissolving 0.5 g gallic acid in 10 mL of ethanol in a 100 mL volumetric flask and diluting to volume with double distilled water. Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 mL of double distilled water. After boiling and subsequent cooling of the solution, a few crystals of sodium carbonate were added. The solution was stand for 24 h, filtered and volume was raised to 1 L with double distilled water. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of stock solution of phenol were added into 100 mL volumetric flask separately and then diluted to volume with double distilled water. The resultant solutions contained concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid. From each calibration solution and sample or blank, 40 µL were pipetted into separate cuvettes and to each 3.16 mL of double distilled water was added. Folin-Ciocalteu's reagent (200 µL) was added and mixed well. After 8 min, 600 µL of sodium carbonate solution was mixed thoroughly in the solution. The solution was allowed to stand at 40 ºC for 0.5 h and absorbance of each solution was noted at 765 nm against the blank (without phenolic solution). A concentration *versus* absorbance linear plot was thus obtained. The concentration of total phenolic compounds of each plant extract in milligram of gallic acid equivalent (GAE)), was determined by using the following standard equation.

$$\text{Absorbance} = 0.118x + 0.0824 \text{ [gallic acid (mg/L)]}$$

Ferric reducing antioxidant power: The reducing capacity of plant extract was measured according to the reported method[19]. Freshly prepared FRAP solution contained 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ solution in 40 mM HCl solution and 2.5 mL of 20 mM ferric chloride solution. The mixture was incubated at 37 °C throughout the monitoring period. 3 mL of FRAP reagent was mixed with 100 µL of sample and 300 µL of distilled water. Absorbance readings were taken at 593 nm after every minute for 6 min. Results were compared with standard curve of ferrous sulphate.

2,2′-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay: 2,2′-Diphenyl-1-picrylhydrazyl free radical scavenging activity was found using a previously reported method[20]. Briefly, DPPH solution (3 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions. The reaction progress of the mixture was monitored at 517 nm over a time period of 40 min. Upon appropriate reduction, the purple colour of the solution changed to yellow diphenylpicrylhydrazine. The percentage of the DPPH remaining ($DPPH_{rem}$ %) was calculated as

$$DPPH_{rem}\ (\%) = [DPPH]_{t=t}/[DPPH]_{t=0} \times 100$$

where $[DPPH]_{t=0}$ is the concentration of DPPH radical before reaction with antioxidant samples and $[DPPH]_{t=t}$ is the concentration of DPPH radical after reaction with antioxidant sample at time t. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 517 nm as a function of time (min)

was plotted for each fraction of the samples. EC_{50} value, which is the concentration of a substance that reduces the amount of DPPH radical to half of the original concentration under experimental conditions, was also determined for each fraction.

Total antioxidant activity in linoleic acid emulsion system by ferric thiocyanate method: Total antioxidant activity of aqueous and organic extracts of both the plants was determined according to the method employed by Mitsuda *et al*.[21]. The solution, which contained 100 mL each of neat or diluted plant extract of both the plants in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7). Each solution was then incubated at 37 ºC in sealed bottles, in dark. The solution without added extract was used as blank, while the solutions containing 100 mL (50 mg/20 mL) of trolox was used as positive control. At intervals of 24 h during incubation, 0.1 mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1 mL each of $FeCl_3$ (20 mM in 3.5 % HCl) and thiocyanate solution (30 %) to ethanolic sample, the solution was stirred for 1 min. After incubation for 5 min at 37 ºC, the absorption values (lipid peroxidation values) were taken at 500 nm.

RESULTS AND DISCUSSION

$ABTS^{\bullet+}$ decolourization assay: The ABTS decolourization assay was applied to evaluate *in vitro* radical scavenging activity of extracts of *T. cordifolia*. The reduction potential of the ABTS radical cation is comparable to that of hydroxyl radical produced during metabolic reactions *in vivo*. All the antioxidative components lower in reduction potential than that of the ABTS radical cation can reduce ABTS radical cation solution proportionate to their amount. Trolox equivalent antioxidant capacity values were obtained by comparing the percentage inhibition values of extract samples with the standard trolox curve. Bar graphs are plotted for the TEAC values of each fraction of the sample (Fig. 1). Trolox equivalent antioxidant capacity values ranged from 0.003-7.654 mM of trolox equivalents. Amongst different fractions, 4, 6, 8, 9, 10, 11 and 12 showed higher TEAC values. With few exceptions a general trend of increase in the TEAC value with increasing polarity of extractive solvent may be seen in Fig. 1. The fractions with less polar extractive solvent showed small TEAC values indicating low solubility of phenolic and other antioxidant components in non-polar medium.

Total phenolic contents: Follin-Ciocalteu's reagent (FC reagent) is usually used in the laboratories for the assessment of phenolic constituents in the plant/herbal extracts and other samples. Hydroxyl moieties of phenolic compounds have the ability to reduce yellow coloured FC reagent to blue colour. The change in the colour is monitored spectrophotometrically at 765 nm. All the fractions showed high values of TPCs (Table-1). Total phenolic contents values ranged from 4.84-107.27 mg of GAE/100 g of dry weight. The high TEAC values obtained by using ABTS radical cation decolourization assay can be attributed to the presence of phenolic or polyhydroxyl moieties of antioxidant components. However no significant

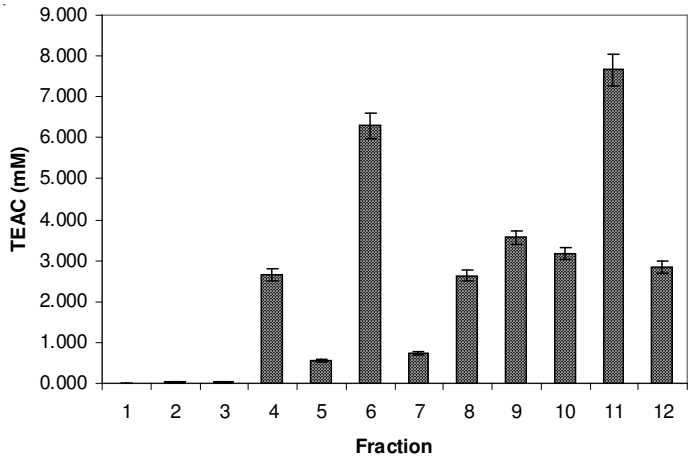

Fig. 1. Comparative profile of TEAC values of different fractions of *T. cordifolia* using the ABTS radical cation decolourization assay

correlation (r^2 = 0.652) between phenolic contents and TEAC values was found. This may be justified by the fact that total phenolic contents alone do not represent the total charge of antioxidants. Further the synergistic effects amongst different antioxidants in the sample may also be accounted for the non-significant correlation. A considerable amount of flavonoid contents of the leaf extracts of *T. cordifolia* (32.1 ± 1.5-45.5 ± 3.5 mg/g of dry solid) was reported by Sengupta *et al.*[22].

TABLE-1
TOTAL PHENOLIC CONTENT (mg OF GAE/100 G OF DRY WEIGHT) OF DIFFERENT FRACTIONS OF *T. cordifolia* BY FOLLIN-CIOCALTEU'S METHOD

Fr. #	Total phenolic content
1	4.84 ± 0.21
2	12.50 ± 0.36
3	17.84 ± 0.52
4	21.32 ± 0.43
5	29.68 ± 0.79
6	67.50 ± 1.12
7	17.42 ± 0.38
8	47.89 ± 0.91
9	65.93 ± 0.83
10	35.23 ± 0.64
11	107.27 ± 1.79
12	97.42 ± 1.31

Note: Data are presented as mean value ± SD (n = 3).

Ferric reducing antioxidant power (FRAP): The FRAP assay as developed by Benzie and Strain[19] which involves a single electron reduction of the $Fe(TPTZ)_2$(III) complex (pale yellow) to the $Fe(TPTZ)_2$(II) complex (blue) by sample antioxidants at acidic pH. Any antioxidant species with lower reduction potential than that of Fe(III)TPTZ salt (0.7 V) can reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ

contributing to FRAP value[23]. This reduction is monitored spectrophotometrically at 593 nm. Appearance of intense blue colouration indicates reducing components in the sample. The original method of Benzie and Strain[19] uses a 4 min interval but we noted that the reaction/colour change is in progress even after 4 min interval. Absorbance readings, therefore, were taken at a 6 min interval after addition of sample to TPTZ reagent allowing the reaction to reach a steady state. The FRAP values of the fractions of *T. cordifolia* were measured by way of comparison with a calibration curve obtained using iron(II) sulfate as the standard reductant (Fig. 2). Ferric reducing antioxidant power values for different fraction ranged from 126.88-1516.80 mg/L of $FeSO_4$ equivalents. Higher FRAP values were obtained for the samples extracted in more polar solvents. It is evident from Fig. 2 that the polarity of the extractive solvent has great influence on the extraction of antioxidant compounds. The order of FRAP values followed the same pattern as that of the samples with increasing polarity. High FRAP values obtained for fractions 4, 5, 7, 8, 9, 11 and 12 may be ascribed partially to the presence of phenolic and flavonoid components. However, coefficient of correlation (r^2) between phenolic compounds and FRAP values was found equal to 0.686.

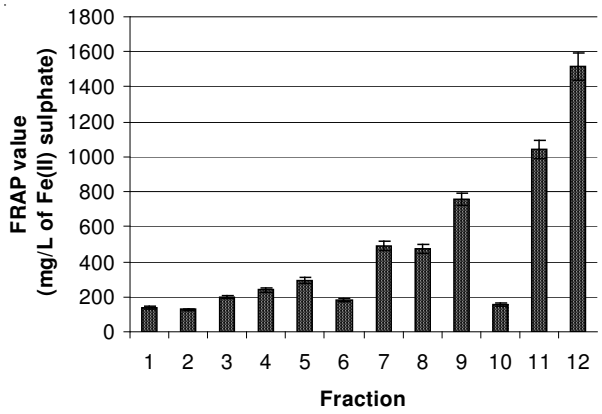

Fig. 2. A comparison of reducing powers in terms of FRAP value of different fractions of *Tinospora cordifolia*

2,2′-Diphenyl-1-picrylhydrazyl radical scavenging assay: DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples. 2,2′-Diphenyl-1-picrylhydrazyl radical scavenging assay was performed by taking parameters, the concentration of antioxidant and the time for completion of the reaction of antioxidants, into consideration. Kinetic curves obtained by plotting DPPH % remaining against time showed that all the fractions (except Fr. 1-4) of *T. cordifolia* contained high levels of DPPH radical scavenging agents (Fig. 3). It is clear from the figure that the curves obtained are much steeper in the first 15 min, showing fast reaction of antioxidant componets with DPPH radical. After 15-20 min the slope of the curves falls sharply and the curve almost becomes parallel to

the time axis showing the completion of most of the antioxidant agents. The major reduction in the absorbance within first 15 min shows that the majority of the antioxidant components present in the samples is fast-reacting. The slow and steady decrease in the absorbance even after 0.5 h indicates the presence of some slow-reacting antioxidant components in the samples. EC_{50} value, which is the concentration of a substance that reduces the amount of DPPH radical to half of the original concentration under experimental conditions, ranged from 83.26-588.31 µg of dried weight (DW)/mL of DPPH solution for Fr. 6 and Fr. 10, respectively (Table-2). On the basis of the data obtained, Fr. 6 may be regarded as the most potent antioxidant fraction, which in turn also proves it to be the best extracting medium for *T. cordifolia* antioxidant components.

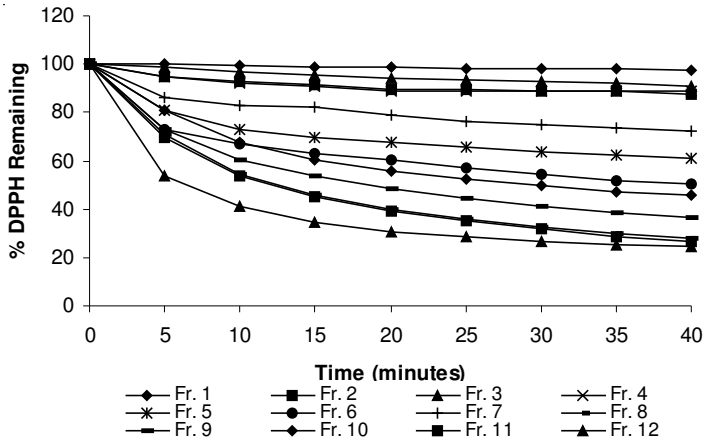

Fig. 3. Time course of DPPH radical scavenging activity of different fractions of *Tinospora cordifolia*

TABLE-2
EC_{50} (AT $T_{30\ min}$) VALUE (µg/mL) FOR DIFFERENT FRACTIONS OF CHLOROFORM EXTRACTS OF *Tinospora cordifolia* USING THE DPPH ASSAY

Fr. #	EC_{50} (at $T_{30\ min}$)
1	n.d.*
2	n.d.*
3	n.d.*
4	n.d.*
5	98.06
6	83.26
7	277.84
8	254.32
9	289.79
10	588.31
11	348.16
12	168.26

n.d.*: not detectable.

Total antioxidant activity in linoleic acid emulsion systems by ferric thiocyanate method: The thiocyanate method was employed to determine the $ROO^{\bullet}$ scavenging activity. The $ROO^{\bullet}$ formed due to oxidation of linoleic acid have the ability to oxidize Fe^{2+}-Fe^{3+}. The peroxidation value is noted spectrophotometrically as increase in the absorbance at 500 nm due to formation of a complex of iron(III) ions with thiocyanate ions (SCN^{-}). Absence of antioxidant agents causes an unrestricted rise in the absorbance of blank. In case of standard or sample solutions, the antioxidants will try to inhibit or slow down oxidation of linoleic acid and will therefore, result into low peroxidation value. Thus a low peroxidation value indicates its capacity to inhibit peroxidation of linoleic acid and *vice versa*. Lipid peroxidation values of different fractions of *T. cordifolia* were determined as a function of time. Trolox was used as positive control. Extract of *T. cordifolia* has been shown to inhibit the lipid peroxidation and superoxide and hydroxyl radicals *in vitro*. Present results support the reported findings as all the fractions of the plants exhibited peroxyl radicals scavenging activity comparable to trolox (Fig. 4).

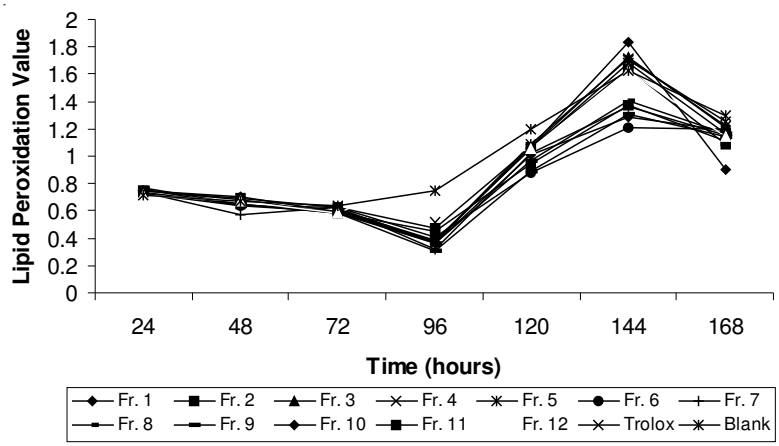

Fig. 4. Time dependent lipid peroxidation values of different fractions of *Tinospora cordifolia*

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