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In vitro Antioxidant and Free Radical Scavenging Activity of the Methanolic Extract of *Tinospora tomentosa* Miers

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ABSTRACT:

Oxidation prevention capability of plant derived extracts could be evaluated by various methods. A promising method is inhibition of lipid decomposition due to peroxidative process, which is nothing but inihibition of oxidative deterioration of lipoidal substances that results in the generation of toxic byproducts including malonyldialdehyde (MDA), hydroxyl alkenals like 4-hydroxy-2-nonenal (4-HNE), etc. play significant roles in the emergence of several illnesses like diabetes mellitus, cancer, atherosclerosis, etc. Considering the aforementioned information, the intention of this study was to assess the antioxidant potential of several extracts made from Tinospra tomentosa Miers.' dried stem (Menispermaceae Family) by observing its effects on ceftriaxone, a cephalosporin antibiotic, mediated peroxidation of lipids taking MDA as a laboratory marker. The study was conducted using liver homogenate of the goat as a lipid source. Different portions of liver homogenate were treated with drug and/or methanolic extract of the plant. After specified hours of incubation level of MDA, 4-HNE, and reduced glutathione (GSH) of different samples was estimated and compared to the control. The study showed that the extract has the capability to check the cephalosporin-mediated peroxidation of lipids significantly. Thus, from the study, we may conclude that the methanolic stem extract of the plant *Tinospora tomentosa* Miers has antioxidant activity. Further studies may be done with the extracts on those ailments that have a link with the accumulation of free radicals.

Keywords:
Lipid peroxidation; ***Tinospora tomentosa*** Miers; Antioxidant; Free Radical Scavengers

Introduction

The role of free radical reactions in disease pathology is well established. This shows that in general, metabolism depends on these interactions but can be detrimental to health as well, including the outcome of various diseases like diabetes, immunosuppression, neurodegenerative diseases, and others. Free radicals lead to cellular necrosis, which is implicated in some membrane pathophysiological conditions, including atherosclerosis, rheumatoid arthritis as well as toxicity of many xenobiotics[1-3].

The term lipid peroxidation[4] has been defined by A.L. Tappel as "the oxidative deterioration of polyunsaturated lipids", i.e., lipids containing multiple carbon- carbon double covalent bonds. This can be considered as a molecular mechanism of cell injury occurring in biomembranes with potential injurious consequences[5].

Lipid peroxidation is a complex process known to occur in biological membranes of both plants and animals that are composed of polyunsaturated fatty acids blended with additional components. Though it can occur invariably in almost all cells of living organisms, the microsomal membranes are more prone to lipid peroxidation owing to the existence of higher concentrations of polyunsaturated fatty acids. It may also occur in fats, oils, paints, plastics, rubbers and causes rancidity or deterioration[6].

Tinospora tomentosa Miers, a large deciduous climbing shrub that can reach an altitude of 1000 meters, is found in tropical thickets in Bengal and nearly all of India. It belongs to the Menispermaceae family. It has a long history of use in Ayurvedic medicine and is referred to locally as "padma-gulancha." The various parts, including the stems, leaves, and roots, have historically been used for a variety of purposes, including hepatic

stimulant, antigout, analgesic, antipyretic, anti-inflammatory, stomachic, bitter tonic, anti-periodic, mild diuretic, emetic, and anti-purgative[7-11]. Numerous studies demonstrate the presence of different phytochemical constituents, primarily sugars, tannins, alkaloids, and flavonoids. According to these studies, the plant includes a variety of pharmacological effects and medicinally active compounds. As a result, the plant promotes the discovery of new therapeutic pathways.

Flavonoids are natural products and are shown to possess antioxidant properties[7]. As *Tinospora tomentosa* contains large amounts of flavonoids it is deemed worthwhile to conduct a scientific investigation into the plant's stems' antioxidant activity.

Materials and methods

The materials used in the study were procured from reputed manufacturers. All the reagents used in the study are of analytical grade and are procured from renowned manufacturers.

The current study's goal was to ascertain *Tinospora tomentosa* Miers' (TTME) methanolic extract's capacity to suppress oxidation. The plant's methanolic extract at various concentrations was tested for its ability to scavenge free radicals, suppress oxidative reactions, and show reducing power.

Ferric thiocyanate pathway for estimating antioxidant activity

The oxidation suppression ability of the methanolic extract of the plant was estimated by adopting ferric thiocyanate technique[12]. In this process, an amount of 10 mg of each extract was dissolved in 1.5 ml of ethyl alcohol. From this 1.25 ml of each solution was pipetted into a reaction mixture containing 2.5 ml of 2.5% linoleic acid and 6.25 ml of 40 mM phosphate buffer (pH 7.0) in a vial. The vials were incubated at 40^{o}C for 60 hours. After incubation 1.25 ml of each vial content was diluted with a quantity of 8.55ml of 75% ethanol, 0.1ml of 30% ammonium thiocyanate and 0.1 ml of 0.1% ferric chloride solution. After thorough mixing, the absorbance of samples was measured at 500 nm and the percent of inhibition was determined. Three different concentrations of the extract were screened for the activity determination by pipetting out 1.25, 2.5, and 5.0 ml of the solution from each vial after the incubation period. Ethanol, without sample, was used as a negative control and butylatedhydroxyanisole was used as a positive control.

$$\text{(capacity to inhibit the peroxide formation in linoleic acid) \% inhibition} = 1 - \frac{\text{(absorbance of sample at 500nm)}}{\text{(absorbance of control at 500nm)}} \times 100$$

A high % inhibition indicates a high antioxidant activity.

Evaluation of reducing power

The reducing power of the methanolic extract of the plant was estimated by Oyaizu method[13]. The extract (100, 500, and 1000 µg) in 1ml of distilled water was combined with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). Incubation of the mixture was done at 50 ^oC for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (2.5 ml) was mixed with distilled water (2.58 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. As a standard, α-tocopherol was used here.

Evaluation of free radical suppressing potential

The free radical suppressing potential of the methanolic extract of the plant was estimated by assessing ceftriaxone-induced lipid peroxidation inhibition. Such inhibition was determined by spectrophotometric method using laboratory markers such as malonyldialdehyde (MDA), reduced glutathione (GSH) and 4-hydroxy-2-nonenal (4-HNE).

i. **Estimation of peroxidation of lipid measured as MDA by 2-thiobarbituric acid (TBA) method:** As MDA serves as a parameter of lipid peroxidation, various techniques have been suggested for its measurement. To estimate lipid peroxidation, the standard curve method was utilized[14]. A certain weight (100 g) of goat liver was properly mixed with a quantity of 100 ml phosphate buffer (pH 8.0) in a mixer grinder. The mixture was divided in equal volume in the corresponding stoppered conical flasks marked as C, (control), D(ceftriaxone sodium treated), D+E500 (ceftriaxone sodium and 500 mg extract treated), D+ E1000(ceftriaxone sodium and 1000 mg extract treated), E500, E1000, D+ A500(ceftriaxone sodium and 500 mg ascorbic acid treated), A500 (500 mg of ascorbic acid treated). Definite weight of drug (ceftriaxone sodium, 2 g/1500 g of liver), methanolic extract (500 and 1000 mg/ 1.5 kg liver) and ascorbic acid (standard, 500 mg / 1.5 kg liver) were added in the respective flasks and shaken for 2 hours in mechanical stirrer. Then 4 ml of mixture homogenate were taken from conical flasks to the respective centrifuge tubes and 4 ml of trichloroacetic acid (TCA) solution (10%w/v, pH 1.2) was added in each tube and mixed well. Then the content was centrifuged at 4000 rpm for about 20 minutes. The supernatant was filtered in respective test tubes. 2 ml of the filtrates were taken directly into corresponding glass stoppered tubes and 2 ml of TBA solution (0.002M) was added in each. All the tubes were placed in a boiling water bath for 30 minutes with a close stopper along with a blank (2 ml TBA+ 2 ml double distilled water). After cooling absorbance was taken at 530 nm against blank. The readings were repeated after 24 hours of incubation.

ii. Estimation of peroxidation of lipid measured as reduced glutathione (GSH) by Ell man's method: Glutathione (GSH) or reduced glutathione is an antioxidant present in all types of living cells. Due to lipid peroxidation, its level decreases. So, estimation of reduced glutathione level is also a way to measure the degree of lipid peroxidation. To estimate reduced glutathione level, the standard curve method was utilized[15]. 1ml of the filtrate was taken directly into the corresponding glass stoppered tubes, and 3 ml of 5,5/ dithiobis (2-nitrobenzoic acid) (DTNB) solution (0.01% in 0.1 N phosphate buffer, pH 8.0) was added in each tube and mixed well. The absorbance was measured immediately at 412 nm against a blank (3 ml DTNB +1 ml double-distilled water). The procedure was repeated after 24 hours of incubation.

iii. **Estimation of lipid peroxidation measured as 4-HNE by spectrophotometric method:** 4-HNE estimation can be used as an index to monitor overall peroxidation of PUFA since 4-HNE is a known product of PUFA oxidative reaction. To estimate lipid peroxidation, the standard curve method was utilized[16]. 1ml of the filtrate was taken directly into corresponding glass centrifuge tubes and 1ml of 2,4-dinitrophenylhydrazine (DNPH) reagent (5 nM) was added in each tube. All the tubes were incubated for 1 hour at room temperature. Then the extraction of these was done with hexane three times and the hexane was evaporated. 2 ml of methyl alcohol was poured into each tube and shaken well and absorbance was taken at 350 nm against methanol as blank. The procedure was also repeated after 24 hours of incubation.

Results and discussion:

The methanolic extract of *Tinospora tomentosa* Miers. exhibited effective antioxidative activity. Estimation of this activity was done by the thiocyanate method. The impact of methanol extract on the peroxidation of linoleic acid is shown in **Figure 1**. The antioxidative potential of the extract is listed in **Table 1**, which indicates that the methanolic extract exhibited stronger antioxidant activity than that of BHA, and the result was found to be significant statistically ($p<0.01$). The more absorbance is an indication of more the concentration of formed peroxides. Whereas the low absorbance indicates high antiperoxidative activity.

The reducing power of Tinospora tomentosa Miers extract is demonstrated in **Figure 2** when compared to α-tocopherol. Using the Oyaizu method, we examined the Fe^{3+} to Fe^{2+} transformation in the presence of the extract samples in order to measure the reductive ability. One important indicator of a compound's probable antioxidant activity is its capacity to reduce[17]. Similar to its antioxidant activity, the plant extract's reducing power rose as the amount of extract increased (**Table 2**). The plant extract demonstrated a potent reducing power that is nearly identical to that of α-tocopherol.

The extract's capacity to scavenge free radicals was ascertained by inhibition of ceftriaxone (a cephalosporin antibiotic) mediated peroxidation of lipids. The outcome is shown in respective figures **(Figure 3-5)** and tables **(Table 3-5**). From the results, it is clear that after 2 hours and 24 hours of incubation, the contents of MDA and 4-HNE in the cephalosporin-treated sample is higher and the GSH content is lower than in the control sample. This indicates that the drug ceftriaxone sodium has lipid peroxidation-inducing capacity. On the other hand, in the drug and antioxidant treated samples the levels of MDA and 4-HNE are less and the level of GSH is more compared to the drug treated samples. This indicates that the methanol extract of the plant has the ability to inhibit drug-induced lipid peroxidation. These outcomes are contrasted with a known antioxidant (ascorbic acid) in all the cases. Results of percent change in MDA, GSH, and 4-HNE profile in drug, drug plus extract-treated, and only extract-treated samples compared to the control show that the drug has induced lipid peroxidation significantly ($t>2$) and the extract can reduce peroxidation significantly ($t>2$).

Conclusion:

The methanolic extract of *Tinospora tomentosa* Miers. demonstrated substantial antioxidant properties, surpassing even standard antioxidants like BHA and nearly matching the efficacy of α-tocopherol in reducing oxidative stress. The extract showed strong free radical scavenging activity, significant reducing power, and the ability to inhibit drug-induced lipid peroxidation. These findings suggest that *Tinospora tomentosa* possesses potent antioxidative and protective effects against oxidative damage, highlighting its potential as a natural antioxidant agent for mitigating lipid peroxidation-related cellular damage and drug toxicities.

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Table 1. *Tinospora tomentosa* Miers. methanol extract's antioxidant activities after 60 hours of incubation

Treatment	Dose (µg)	Absorbance (nm)	% Inhibition
TTME	125	0.452 ± 0.29	57.35 ± 1.370*
	250	0.263 ± 0.16	75.18 ± 0.740*
	500	0.098 ± 0.21	90.75 ± 1.100*
BHA	125	0.007 ± 0.002	99.43 ± 0.212*
	250	0.004 ± 0.019	99.62 ± 0.023*
	500	0.002 ± 0.001	99.81 ± 0.020*

TTME- Methanol extract of *Tinospora tomentosa* Miers., BHA (Standard)- Butylatedhydroxyanisole.*P<0.01 by using Dunnett's test after a one-way ANOVA

Table 2. Reducing power of methanol extract of *Tinospora tomentosa* Miers. after 60 hours of incubation

Treatment	Concentration (µg/ml)	Absorbance (nm)
TTME	100	0.215 ± 0.008
	500	0.324 ± 0.006*
	1000	0.458 ± 0.04*

STD	100	0.207 ± 0.003*
	500	0.364 ± 0.004*
	1000	0.479 ± 0.046*

TTME- Methanol extract of *Tinospora tomentosa* Miers., STD (Standard)- α- tocopherol.*P<0.01 by using Dunnett's test after a one-way ANOVA

Table 3. Percentage change in MDA profile (Methanol extract of *Tinospora tomentosa* Miers.)

Time period	**Animal set**	**D**		**D+E$_{500}$**		**D+E$_{1000}$**		**D+A$_{500}$**		**E$_{500}$**		**E$_{1000}$**		**A$_{500}$**	
		% Change	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**
2 hrs.	1.1	18.63	3.64	10.88	7.12	8.49	11.42	8.66	2.89	-1.37	4.36	-2.17	11.76	-4.36	6.50
	1.2	20.65	7.67	11.29	8.32	9.04	6.96	9.48	5.60	-0.96	11.62	-1.86	14.35	-3.41	11.41
	1.3	20.85	6.94	13.17	6.12	8.56	8.41	10.24	18.44	-1.24	14.49	-1.72	16.29	-5.12	17.34
	1.4	19.68	8.62	12.24	8.40	9.18	19.32	9.56	8.23	-1.76	10.42	-2.03	8.85	-4.73	12.32
	1.5	21.40	7.60	13.68	7.86	10.24	16.65	10.18	6.62	-1.84	19.32	-1.98	7.39	-3.28	8.78
		20.24± 0.48		12.25± 0.49		9.10± 0.31		8.62± 0.28		-1.43± 0.16		-1.95± 0.07		-4.18± 0.35	
24 hrs.	1.1	14.64	12.02	8.06	4.28	6.86	17.32	10.64	7.46	-0.88	7.84	-0.96	13.79	-1.49	7.88
	1.2	17.75	7.36	9.48	6.14	7.42	12.46	11.21	2.94	-0.67	11.26	-1.23	17.38	-1.68	12.44
	1.3	17.48	6.72	11.56	6.26	6.67	13.32	10.71	16.56	-0.92	15.45	-1.41	12.18	-2.02	16.69
	1.4	12.37	5.23	9.78	4.11	7.69	17.02	9.88	11.42	-0.84	10.18	-1.07	9.46	-1.46	18.12
	1.5	15.24	9.44	10.62	3.24	8.04	8.96	10.26	6.48	-1.02	12.42	-1.28	11.21	-.094	15.43
		15.49± 1.43		9.90± 0.58		7.33± 0.25		6.54± 0.22		-0.86± 0.05		-1.19± 0.07		-1.34± 0.32	

Table 4. Percentage change in GSH profile (Methanol extract of *Tinospora tomentosa* Miers.)

Time period	**Animal set**	**D**		**D+E$_{500}$**		**D+E$_{1000}$**		**D+A$_{500}$**		**E$_{500}$**		**E$_{1000}$**		**A$_{500}$**	
		% Change	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**
2 hrs.	1.1	-18.18	8.65	-16.42	26.14	-11.41	17.32	-4.87	4.41	1.16	3.37	1.94	6.36	4.14	21.33
	1.2	-23.07	9.24	-14.89	18.39	-11.02	12.16	-5.82	7.95	1.70	5.93	2.08	8.19	5.02	16.79
	1.3	-25.80	6.78	-16.47	14.32	-10.05	10.72	-6.08	9.21	1.28	8.14	2.25	11.47	2.04	20.18
	1.4	-33.03	3.84	-24.66	19.65	-14.83	13.47	-3.95	11.34	1.34	10.36	2.41	10.44	2.31	19.92
	1.5	-18.48	11.26	-11.36	12.37	-9.39	11.14	-4.98	16.33	2.42	15.19	3.12	16.35	4.69	18.45
		-23.71 ±2.73		-16.76 ±2.18		-11.34 ±0.83		-5.14 ±0.37		1.58 ±0.22		2.36 ±0.20		3.64 ±0.61	
24 hrs.	1.1	-22.24	14.71	-19.39	12.46	-13.91	11.32	-2.76	7.31	1.20	3.36	1.71	4.42	2.21	20.11
	1.2	-24.18	11.39	-16.46	18.30	-12.42	14.39	-3.04	6.10	1.01	4.98	1.43	7.90	1.87	18.15
	1.3	-27.76	16.71	-18.10	16.13	-14.19	16.76	-4.18	11.17	0.92	6.15	1.20	8.65	3.01	12.39
	1.4	-36.70	10.35	-27.40	10.19	-18.11	8.12	-3.44	16.32	0.86	7.92	1.06	10.14	1.64	8.45
	1.5	-21.02	12.73	-13.35	18.41	-10.17	10.49	-5.38	12.41	1.21	11.05	1.50	12.74	2.57	13.62
		-26.38 ±2.81		-18.94 ±2.79		-13.76 ±1.29		-3.76 ±0.46		1.04 ±0.06		1.38 ±0.11		2.26 ±0.24	

Table 5. Percentage Change in 4-HNE profile (Methanol extract of *Tinospora tomentosa* Miers.)

Time period	**Animal set**	**D**		**D+E$_{500}$**		**D+E$_{1000}$**		**D+A$_{500}$**		**E$_{500}$**		**E$_{1000}$**		**A$_{500}$**	
		% Change	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**	**% Change**	**t-value**
2 hrs.	1.1	36.97	16.26	26.67	4.65	23.16	26.16	14.97	2.83	-0.95	2.66	-1.82	3.34	-4.66	16.94
	1.2	39.45	12.93	22.28	7.16	17.94	21.38	16.84	6.18	-1.01	4.74	-1.64	5.62	-3.92	26.34
	1.3	36.66	14.92	28.39	9.14	18.12	18.65	18.16	11.92	-1.26	5.23	-1.97	4.11	-2.87	21.91
	1.4	38.14	18.27	23.16	13.35	15.38	8.43	15.91	10.17	-1.54	9.18	-2.94	2.98	-3.45	20.37
	1.5	37.63	21.37	23.50	16.12	18.60	12.14	18.27	16.11	-1.84	6.47	-3.98	6.18	-4.50	12.38
		37.77 ±0.49		24.80 ±1.16		18.64 ±1.26		16.83 ±0.63		-1.32 ±0.16		-2.47 ±0.43		-3.88 ±0.33	
24 hrs.	1.1	30.40	12.32	24.78	4.42	22.31	6.39	19.77	4.14	-0.62	2.24	-1.41	3.36	-3.66	4.98
	1.2	29.73	8.69	21.24	6.94	16.22	11.21	21.29	6.19	-0.79	5.38	-0.98	4.91	-2.74	6.35
	1.3	24.64	12.17	19.66	11.39	14.78	16.15	13.85	11.24	-1.01	6.16	-1.49	8.14	-1.90	11.48
	1.4	21.38	16.25	17.53	19.14	12.39	23.89	11.37	19.11	-0.95	10.27	-1.26	9.26	-2.44	10.16
	1.5	27.65	19.81	16.49	13.46	12.85	16.49	10.82	16.38	-0.83	4.92	-1.56	11.38	-1.11	16.42
		26.76 ±1.67		19.94 ±1.46		15.71 ±1.78		15.42 ±2.16		-0.84 ±0.06		-1.34 ±0.10		-2.37 ±0.42	

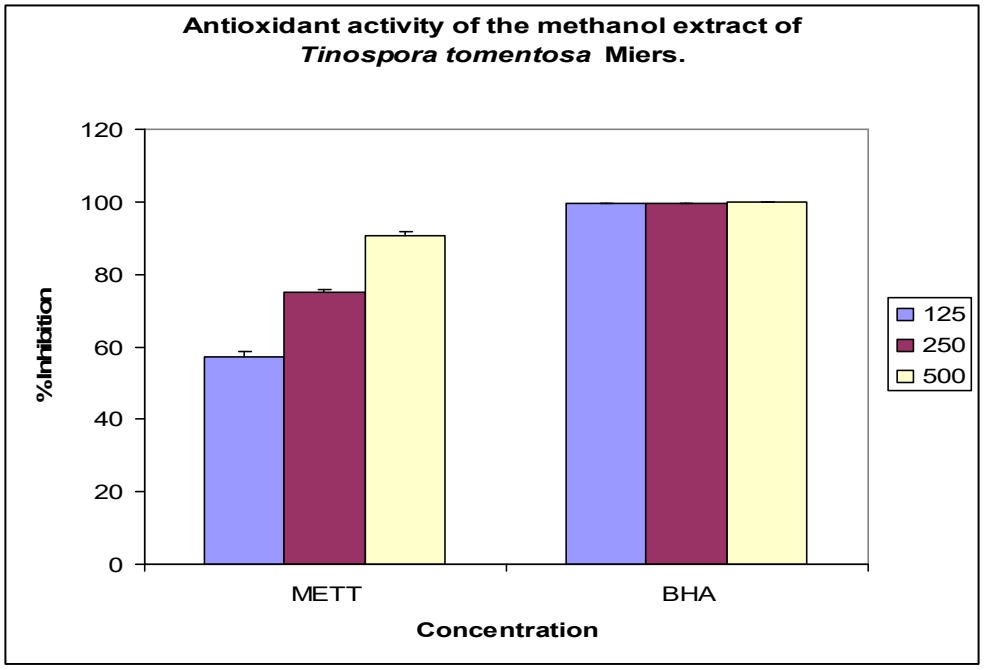

Figure 1. Antioxidant activity of methanol extract of *Tinospora tomentosa* Miers. after 60 hours of incubation

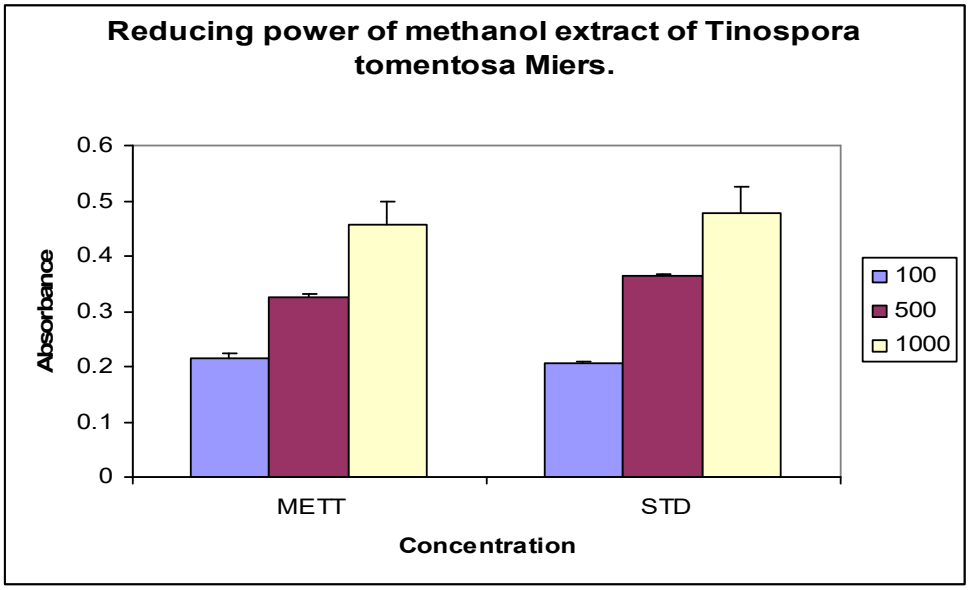

Figure 2. Reducing power of methanol extract of *Tinospora tomentosa* Miers. after 60 hours of incubation

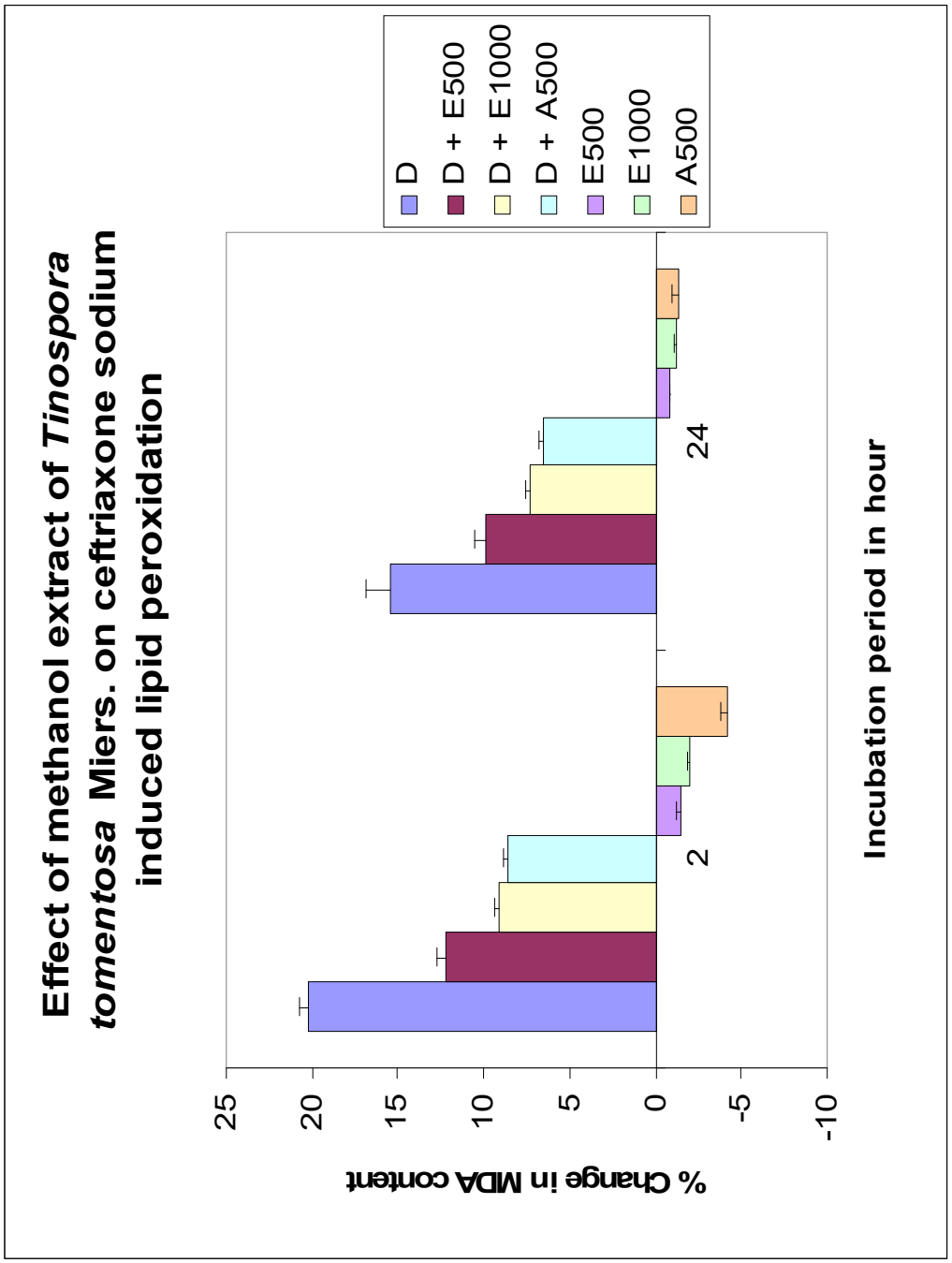

Figure 3. Percentage change in MDA profile
(Methanol extract of *Tinospora tomentosa* Miers.)

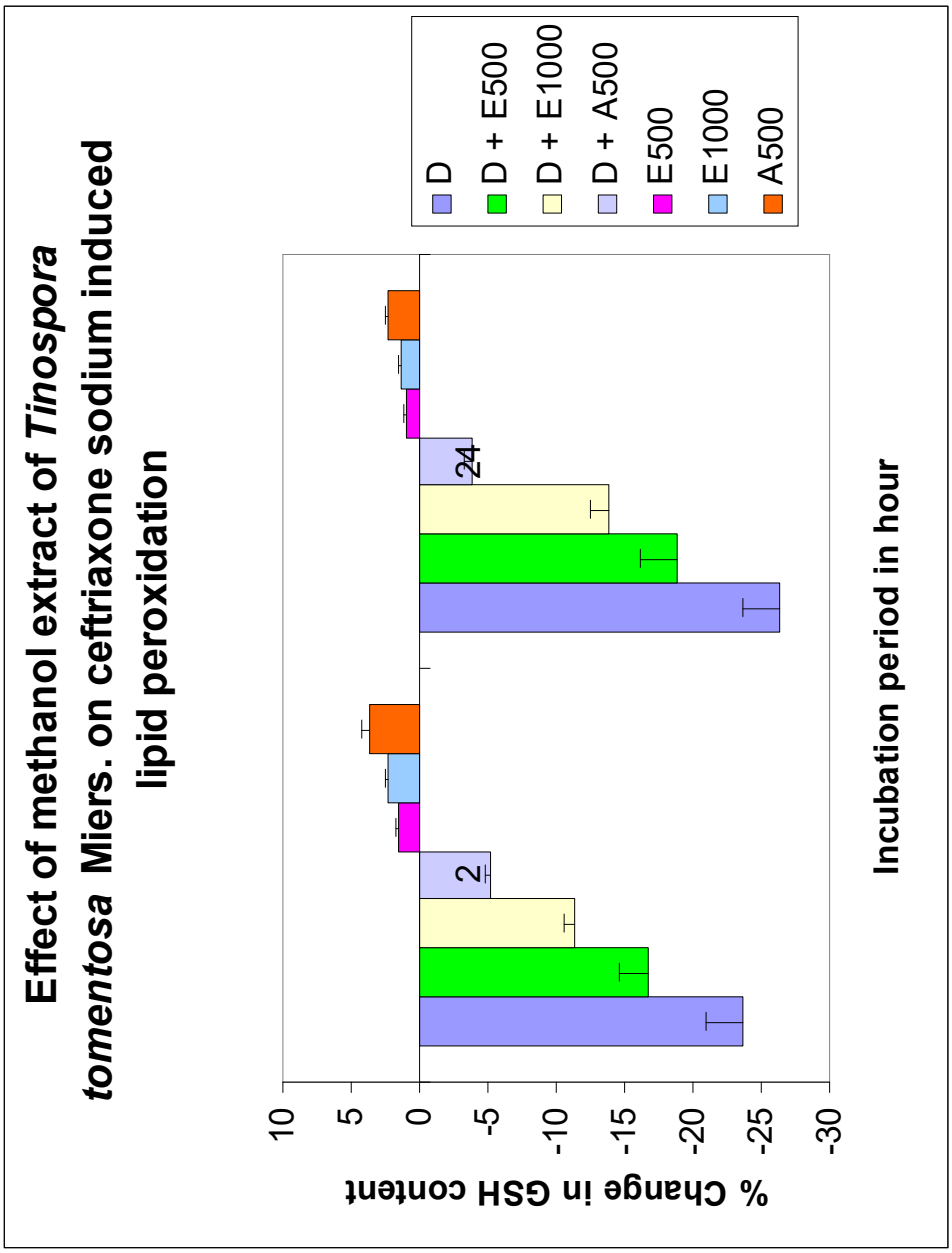

Figure 4. Percentage change in GSH profile
(Methanol extract of *Tinospora tomentosa* Miers.)

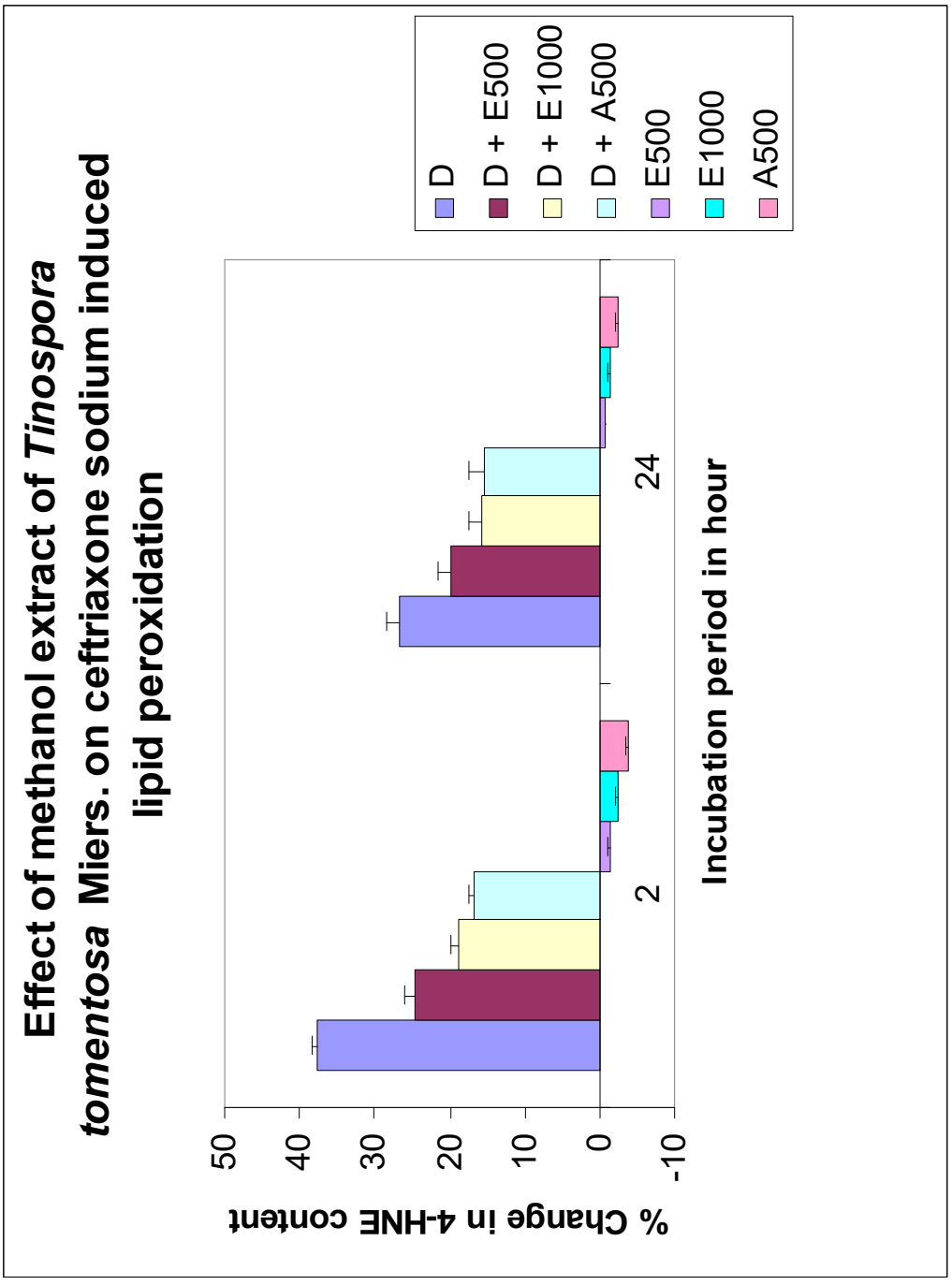

Figure 5. Percentage change in 4-HNE profile (Methanol extract of *Tinospora tomentosa* Miers.)