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ORIGINAL ARTICLE

Oxidative Stress and Modulatory effects of the root extract of *Phlogacanthus tubiflorus* on the activity of Glutathione-S-Transferase in Hydrogen Peroxide treated Lymphocyte

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Glutathione-*S*-transferase is one of the important enzyme systems that plays vital role in decomposition of lipid hydro-peroxides formed due to oxidative stress. In the present study GST activity increased in the lymphocytes treated with increasing concentration of H_2O_2, and decrease in the levels of GSH was observed. For similar treatment conditions LDH activity and MDA levels increased significantly leading to decrease in the cell viability. Treatment of lymphocytes with the root extract of *Phlogacanthus tubiflorus* (PTE) resulted in dose dependent decline in the GST activity and rise in GSH levels. LDH activity and MDA levels also declined that led to the increase of cell viability. Lymphocytes pre-treated with the PTE followed by H_2O_2 (0.1 and 1%) treatment, decline in the activity of GST and increase in GSH levels was observed. Also we have observed decline in the activity of LDH and MDA levels in the lymphocytes for both 0.1 and 1% of H_2O_2 though the magnitude of change was higher in the lymphocytes pre-treated with the PTE followed with 1% of H_2O_2 treatment. Significant increase in the cell viability for similar conditions was also observed. These findings suggest protective function of the root extracts might be through modulation of GST activity and levels of GSH and might find application in Chemomodulation in future.

Key words: Oxidative Stress / Glutathione-S-transferase / Phlogacanthus tubiflorus / Lymphocyte / Chemomodulation.

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Key words: Oxidative Stress / Glutathione-S-transferase / Phlogacanthus tubiflorus / Lymphocyte / Chemomodulation.

Abbreviations: ROS: Reactive Oxygen Species, GST: Glutathione-S-transferase, GSH: Reduced Glutathione, LDH: Lactate Dehydrogenase, LP: Lipid Peroxidation, PTE: Phlogacanthus tubiflorus Extract, PBS: Phosphate Buffer Saline.

Oxidative stress causes the production of free radicals and reactive oxygen species (ROS) in the biological system (Subhashinee et al., 2005). The excessive production of such reactive species results in the imbalance in between oxidants and antioxidants that leads to tissue injuries and

contribute to the progression of the several degenerative diseases in humans, such as coronary heart disease, cataracts, muscle degeneration, ageing and cancer (Caporossi et al., 2003; Tandon et al., 2004; Stocker and Keaney, 2004; Shichi, 2004; Ben-Porath and Weinberge, 2005).

Cellular antioxidant enzyme system scavenges and/or neutralizes reactive oxygen species generated under oxidative stress. Glutathione-*S*-transferase (GSTs) (E.C.2.5.1.18) is one of the important constituents of this system, belongs to the super family of enzymes that plays vital role in decomposition of lipid hydro-peroxides formed due to ROS induced peroxidation of the membrane lipids. GST catalyzes the conjugation of reduced glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds, the first step in mercapturic acid pathway that leads to the elimination of toxic compounds (Habing et al., 1974; Hayes et al., 2005).

GSTs have been implicated in the development of resistance towards chemotherapy agents (Hayes and Paulford, 1995; Danyelle and Kenneth, 2003). It is not surprising that levels of GST are elevated in some tumors cells; this may play a role in drug resistance. The elevated levels of GSTs in human tumors can be promising therapeutics targets for research. Numbers of Synthetic compounds and botanicals like phenolics, flavonoids have been studied in in vitro and in vivo systems as a source of antioxidant to modulates the GST activity and subsequent sensitization of tumor cells to chemotherapeutic agents. But, the modulation of GST activity is not enough to combat with detrimental effects of ROS that causes subsequent lowering of drug resistance in chemotherapy (Tew, 1994; Ruzza et al., 2009; Andrea et al., 2010; Elizabeth and Nira, 2010). Also the molecular events associated with modulation of GST activity and other related systems are poorly understood.

The aim of the present study was to investigate the modulatory effects of the root extracts of *Phlogacanthus tubiflorus* (Family: Acanthaceae) on the activity of the glutathione-*S*-transferase in the lymphocytes, cultured *in vitro*, and exposed to hydrogen peroxide (H_2O_2). The selection of the plant is based on the ethno-pharmacological data obtained by consulting the traditional healer and thereafter consulting the scientific literature related to the identified plant. *Phlogacanthus tubiflorus Nees* is traditionally used by the tribal population of North Eastern Region of India for treating wounds, tumorous growth and also as a blood purifier (Indigenous knowledge). Besides this we have also investigated the levels of reduced glutathione (GSH), lipid peroxidation (LP) and Lactate dehydrogenase (LDH, EC 1.1.1.27) activity and tried to correlate with the GST activity.

MATERIALS AND METHODS

Preparation of Modulator

The roots of *Phlogacanthus tubiflorus* were collected from Tezpur, Assam (India) and were authenticated by a competent Botanist, Prof. S K Borthakur, at the Department of Botany, Gauhati University, Gauhati, Assam (India) and Voucher specimen was preserved in our laboratory. The roots were washed with running tape water repeatedly and finally with distilled water to remove impurities and dried at shade. The dried plant materials were finely powdered and macerated with 80% (v/v) ethanol in a shaking condition. The extract thus obtained (PTE) were filtered and concentrated and stored at 4^0 C. The extract was dissolved in DMSO with final concentration of 2.5 mg/ml.

Isolation of Lymphocytes

Chicken blood was collected from source and was diluted 1:1 with PBS then layered 6 ml into 6 ml Histopaque (1.077 gm/ml). Lymphocytes were isolated from the sample after centrifugation for 30 minutes at 400 g. Lymphocytes were then washed with 2 ml PBS and 2 ml serum free media separately through centrifugation for 10 minutes at 250 g. Cell pellets were then suspended in PBS and cell viability was checked by Trypan blue exclusion method using haemocytometer. Cell viability more than 90 % was used for subsequent study.

Lymphocytes culture and treatment

Aliquots of 200 µl of isolated lymphocytes were seeded in 96 well culture plate in RPMI supplemented with 10% heat inactivated Fetal bovine serum (FBS) and were treated with H_2O_2 / PTE / PTE+H_2O_2 as per experimental requirements and maintained at 37^0C and 5 % CO_2 in CO_2 incubator. Lymphocytes were treated for 4 hours in case of only H_2O_2 and PTE treatment while in other cases pre treated with PTE for 1 hour and then treated with H_2O_2 for 4 hours. After incubation, lymphocytes were centrifuged and washed with PBS, homogenized in PBS. Cell supernatants were used for assaying GST, GSH, Protein and LP while cell free media were used for assaying LDH.

Glutathione-*S*-Transferase

The specific activity of cytosolic GST was determined spectrophotometrically (Habig et al., 1974). In brief, the reaction volume (1 ml) contained final concentration of 0.1 M phosphate buffer (pH 6.5), 1 mM CDNB in 95% ethanol and 1 mM GSH and was incubated at 37^0C for 5 min. The reaction was initiated by the addition of enzyme sample and the activity was measured for 3 min at 340 nm (Cecil Aquarius, 7000 series). The specific activity of GST was calculated using the extinction coefficient 9.6 $mM^{-1}cm^{-1}$ at 340 nm and expressed in terms of percentage change of µ mole of CDNB-GSH conjugates formed/min/mg proteins.

Reduced glutathione

Reduced glutathione content was estimated as the total non-protein sulphydryl group by the standard procedures (Moron et al., 1979). The proteins were precipitated by addition of tricholoroacetic acid (TCA), centrifuged and supernatant was collected. The supernatant was mixed with 0.2 M phosphate buffer (pH 8) and 0.6 M 5,5'-dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer, and allowed to stand for 8-10 min at room temperature. The absorbance was recorded at 412 nm using a spectrophotometer (Thermo Scientific, UV 10). Reduced glutathione (GSH) was used as a standard to calculate nMole of –SH content/mg protein and finally expressed as percentage change of GSH level.

Lactate Dehydrogenase

The specific activity of Lactate dehydrogenase (LDH) released into the medium as a result of membrane damage was assayed by measuring the rate of oxidation of NADH at 340 nm (Bergmeyer and Bernt, 1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM sodium pyruvate, 0.1 mM NADH and required amount cell free media to make the final volume of 1 ml. The reaction was started at 25^0C by addition of NADH and the rate of oxidation of NADH was measured at 340 nm using a spectrophometer (Cecil Aquarius, 7000 series). The enzyme activity was calculated using extinction coefficient 6.22 $mM^{-1}Cm^{-1}$/mg protein and finally expressed as percentage change of LDH activity.

Lipid Peroxidation

Peroxidative damage was estimated spectrophotometrically by the assay of

thiobarbituric acid reactive substances (TBARS) and expressed in terms of nMole of malondoialdehyde (MDA) formed per mg protein (Okhawa et al., 1979). Briefly, in a 3 ml reaction volume cell homogenate supernatant was mixed with 0.15 M Tris–KCl buffer (pH 7.4) and 30% tricholoroacetic (TCA) and 52 mM thiobarbituric acid (TBA). The mixture was heated for 45 minutes at 80^0 C, cooled and centrifuged for 10 minutes at 3000 rpm. The absorbance of the clear supernatant was measured against distilled water blank at 531.8 nm in spectrophotometer (Thermo Scientific, UV 10) and finally expressed as percentage change of nMole of MDA formed per mg protein.

Cell Viability assay

Cell viability assay were performed according to the MTT based method (Denizot and Lang (1986). The key component (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) is yellowish in color and mitochondrial dehydrogenase of viable cells cleave the tetrazolium ring, yielding purple insoluble formazan crystals which were dissolved in suitable solvent. The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material. Briefly, after treatments, lymphocytes were treated with 10 % of MTT for 2 hours and after which formazan crystals were dissolved in solvent and its absorbance were measured at 570 nm. The absorbance of control cells was set as 100% viability and the values of treated cells were calculated as percentage of control.

Protein determination

The protein contents were determined using bovine serum albumin (BSA) as standard (Lowry et al., 1951).

Statistical Analysis

All the data are expressed as means ± SD, n=3. Results were statistically analyzed by student's t test for significance difference between group mean using GraphPad software (Bourke et al., 1985). The significance difference between the experimental and the control group was set at different levels as $p< 0.05$, $p< 0.01$ and $p< 0.001$.

RESULTS

Effects of different concentrations of H_2O_2 (Table 1)

Exposure of lymphocytes to increasing concentration of H_2O_2 (0.1, 0.2, 0.5 and 1%), increase in the activity of GST in dose dependent manner was observed and at 1.0% H_2O_2 treatment GST activity increased to 168.42% ($p<0.01$) in comparison to untreated cells. For similar conditions significant decline in the levels of GSH was observed. Per-oxidation of membrane lipids is indicator of oxidative stress experienced by the cells. Dose dependent increase in the level of lipid peroxidation was observed on treatment with H_2O_2. The increase in the lipid peroxidation was significant for all the dose of H_2O_2 used in the experiment. Further we also observed increase in LDH activity and decline in the cell viability. For 1.0% of H_2O_2 treatment the decline in cell viability was below 50% (Figure 1a).

Effects of the root extract of *Phlogacanthus tubiflorus* (PTE) (Table 2)

Lymphocytes treated with 50, 100 and 200 µg/ml of root extracts of *Phlogacanthus tubiflorus* decrease in the activity of GST was observed. The decrease in the GST activity was significant at 100 and 200 µg/ml of root extracts used in the experiment. In contrast to the GST activity,

significant increase in the levels of GSH was observed. The treatment of the root extracts resulted in the dose dependent decline in the levels and activity of MDA and LDH respectively. For the similar conditions the cell viability also increased in comparison to untreated cells (Figure 1b).

PTE as modulator in the cells treated with 0.1% H_2O_2 treatment

Dose dependent increase in the levels of reduced Glutathione (GSH) was observed in the lymphocytes pre-treated with root extracts followed by 0.1% H_2O_2 treatment for 4 h. For similar conditions decline in the activity of GST was observed. Significant decline in the levels of MDA and activity of LDH respectively were observed. Cell viability increased for all doses of the root extracts used in experiment (Figure 1c).

Root extracts of PTE as modulator in the cells treated with 1% H_2O_2

Lymphocytes were pre-treated with root extracts of PTE for 1h followed by the treatment with 1% H_2O_2 for 4 h, decline in the activity of GST was observed. Glutathione levels increased in dose dependent manner as compared to positive control. For similar conditions decline the levels and activity of MDA and LDH was observed as compared to the positive control but the overall activity and levels were significantly high as compared to the negative control. The cell viability increased for 50, 100 and 200 µg/ml of the root extract used as compared to positive control (Figure 1d).

Table 1: Effects of different concentrations of H_2O_2. Lymphocytes were treated with the indicated concentrations of H_2O_2 for 4h and activity of GST and LDH; and level of GSH and MDA were measured as described in material and method section. Values are mean ± SD; n=3; $^{a}p<0.001$ compared to control cells; $^{b}p<0.01$ compared to control cells; $^{c}p<0.05$ compared to control cells.

Treatments (H_2O_2)	% Change of GST activity (unit/mg protein)	% Change of GSH level (nMole/mg protein)	% Change of LDH activity (unit/mg protein)	% Change of MDA level (nMole/mg protein)
Control	100±7.49	100±8.83	100±7.45	100±4.76
0.10%	108.76±7.25	92.75±4.77	155.79±10.39 ^{b}	110.69±2.79
0.20%	120.97±1.12 ^{b}	86.40±0.64	172.34±11.49 ^{a}	139.63±9.31 ^{b}
0.50%	133.54±8.90 ^{b}	67.05±4.47 ^{b}	217.22±14.48 ^{a}	202.01±2.03 ^{a}
1%	168.42±6.74 ^{b}	45.31±2.79 ^{a}	264.32±17.62 ^{a}	251.55±16.77 ^{a}

Table 2: Effects of the root extracts of *Phlogacanthus tubiflorus* (PTE). Lymphocytes were treated with the indicated concentrations of PTE for 4h and activity of GST and LDH; and level of GSH and MDA were measured as described in material and method section. Values are mean ± SD; n=3; $^{a}p<0.001$ compared to control cells; $^{b}p<0.01$ compared to control cells; $^{c}p<0.05$ compared to control cells.

Treatments (PTE)	% Change of GST activity (unit/mg protein)	% Change of GSH level (nMole/mg protein)	% Change of LDH activity (unit/mg protein)	% Change of MDA level (nMole/mg protein)
Control	100±6.67	100±8.12	100±2.67	100±1.99
50µg/ml	88.04±5.87	119.53±7.97 ^{c}	91.35±6.09	98.52±6.57
100 µg/ml	80.94±5.40 ^{c}	136.68±9.11 ^{b}	66.22±4.42 ^{a}	91.89±6.13
200 µg/ml	72.53±4.84 ^{b}	139.71±9.31 ^{b}	63.15±4.21 ^{a}	76.85±5.12 ^{b}

Table 3: Protective effects of different concentrations of PTE in H_2O_2 treated lymphocytes. Lymphocytes were pre-treated with PTE for 1 h and followed by 0.1% H_2O_2 treatment for 4h and activity of GST and LDH; and level of GSH and MDA were measured as described in material and method section. Values are mean ± SD; n=3; [a]p<0.001 compared to control cells; [b]p<0.01 compared to control cells; [c]p<0.05 compared to control cells; [d]p<0.001 compared to cells treated with only H_2O_2; [e]p<0.01 compared to cells treated with only H_2O_2; [f]p<0.05 compared to cells treated with only H_2O_2.

Treatments	% Change of GST activity (unit/mg protein)	% Change of GSH level (nMole/mg protein)	% Change of LDH activity (unit/mg protein)	% Change of MDA level (nMole/mg protein)
Control	100±6.67	100±6.67	100±6.67	100±6.67
H_2O_2 (0.1%)	111.97±7.46	93.73±6.25	148.00±9.87 [b]	121.34±8.09 [c]
H_2O_2 (0.1%) + PTE(50 µg/ml)	74.55±5.22 [be]	112.53±7.50	132.26±8.82 [b]	115.24±7.68
H_2O_2 (0.1%) + PTE(100 µg/ml)	67.42±4.49 [bd]	127.86±8.52 [ce]	96.26±8.82 [e]	96.88±4.42 [f]
H_2O_2 (0.1%) + PTE(200 µg/ml)	70.85±4.72 [be]	140.57±9.37 [be]	44.70±2.98 [ad]	66.80±4.42 [bd]

Table 4: Protective effects of different concentrations of PTE in H_2O_2 treated lymphocytes. Lymphocytes were pre-treated with PTE for 1 h and followed by 1% H_2O_2 treatment for 4h and and activity of GST and LDH; and level of GSH and MDA were measured as described in material and method section. Values are mean ± SD; n=3; [a]p<0.001 compared to control cells; [b]p<0.01 compared to control cells; [c]p<0.05 compared to control cells; [d]p<0.001 compared to cells treated with only H_2O_2; [e]p<0.01 compared to cells treated with only H_2O_2; [f]p<0.05 compared to cells treated with only H_2O_2.

Treatments	% Change of GST activity (unit/mg protein)	% Change of GSH level (nMole/mg protein)	% Change of LDH activity (unit/mg protein)	% Change of MDA (nMole/mg protein)
Control	100±6.67	100±8.48	100±5.62	100±6.32
H_2O_2 (1%)	143.26±9.55 [b]	46.16±3.08 [a]	280.93±9.87 [a]	257.70±4.16 [a]
H_2O_2 (1%) + PTE(50 µg/ml)	80.40±5.36 [cd]	90.32±6.19 [d]	250.51±16.70 [a]	219.13±14.61 [a]
H_2O_2 (1%) + PTE(100 µg/ml)	72.60±4.84 [bd]	118.66±7.91 [cd]	161.52±10.77 [ad]	212.75±14.18 [ae]
H_2O_2 (1%) + PTE(200 µg/ml)	79.92±5.33 [cd]	142.48±9.50 [bd]	123.04±13.52 [d]	196.5±13.09 [ae]

DISCUSSION

Here in the present study dose dependent increase in the GST activity and decline in the levels of glutathione was found in lymphocytes treated with increasing concentration of H_2O_2. GSTs are present in many organs and have been implicated in the detoxification of endogenous α,β unsaturated aldehydes formed during lipid peroxidation induced by oxidative damage (Esterbaucer et al.,1991) . The increase in the GST activity is due to the formation of lipid peroxides in the cells on H_2O_2 treatment. This is indicated by increase in the levels of MDA formation in the cell (Milei et al., 2007) (Table 1). GSH is co factor of GST and is responsible for the redox status of cell. The decline in the levels of GSH is due to increase in the activity of GST. The significant increase in the levels of lipid peroxides

measured as MDA formation has led to significant fall in the cell viability suggesting the cellular damage at all concentration of H_2O_2 treatment. This is also indicated by the increase in the activity of LDH released in to the media (Goswami et al., 2003; Kim et al., 2008).

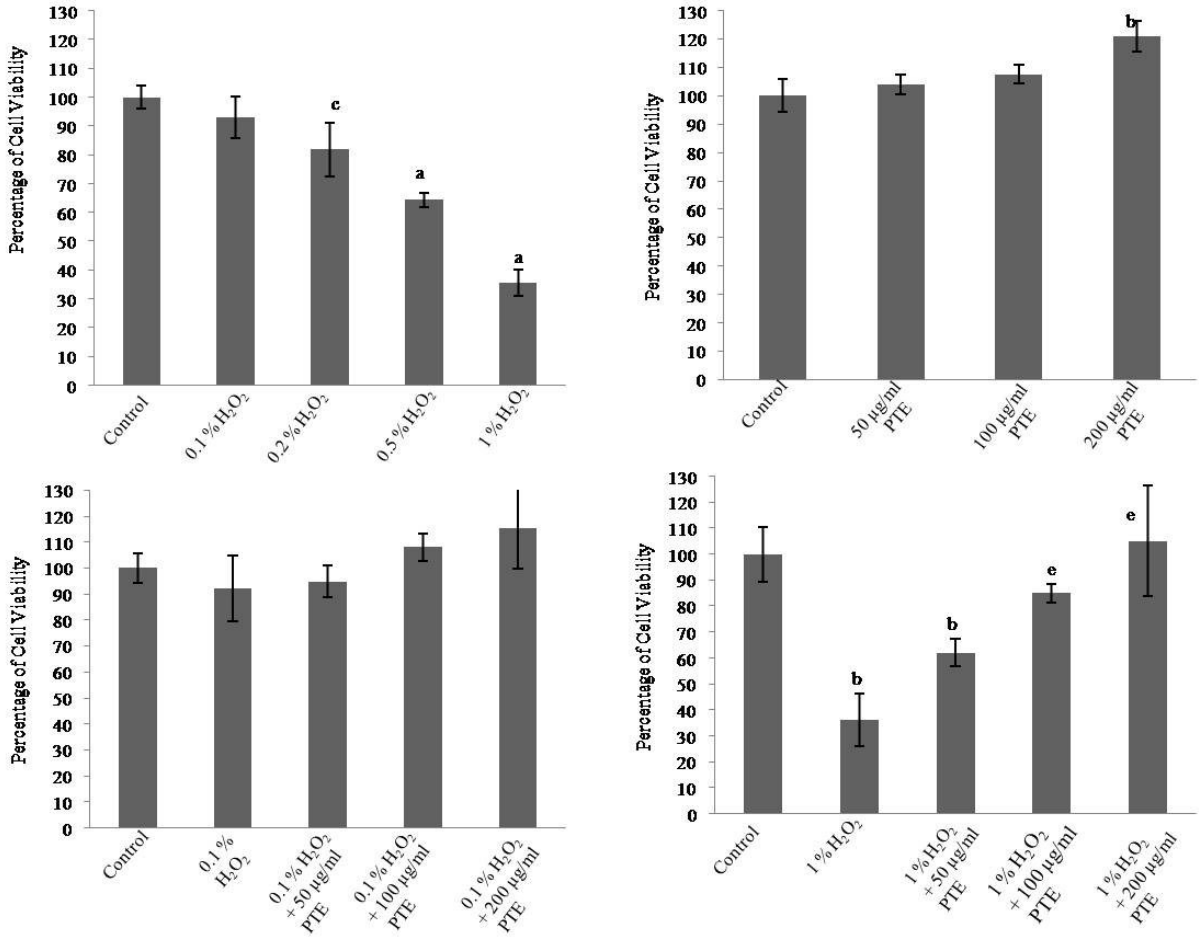

Figure 1: Results Cell Viability measured by MTT Assay. (a) Effects of different concentration of H_2O_2; (b) Effects of different concentration of PTE; (c) Pre-exposure of cells with PTE followed by 0.1 % H_2O_2; (d) Pre-exposure of cells with PTE followed by 1% H_2O_2 treatment. Values are mean ± SD; n=3; $^{a}p<0.001$ compared to control cells; $^{b}p<0.01$ compared to control cells; $^{c}p<0.05$ compared to control cells; $^{d}p<0.001$ compared to cells treated with only H_2O_2; $^{e}p<0.01$ compared to cells treated with only H_2O_2; $^{f}p<0.05$ compared to cells treated with only H_2O_2.

When the lymphocytes were treated with root extracts of *Phlogacanthus tubiflorus*, decline in the activity of GST and increase in the levels of GSH was observed. As GST is involved in the detoxification of lipid peroxides, significant fall in the levels of lipid peroxidation might have led to decline in the activity of GST. In contrast the cell viability increased significantly with the treatment of the root extracts (figure 1b), this is indicated from the decline in the LDH activity for similar conditions. This finding suggests the antioxidant property of root extracts as the treatment has led to the dose dependent decrease in the lipid peroxide levels and significant modulation of GSH levels and GST activity (Saravanan et al., 2003; Dahiru et al., 2005; Pardhasarathi et. al., 2005). This might have resulted in the significant increase in the cell viability.

In the lymphocytes pre-treated with the root extracts 50, 100 and 200 µg/ml for 1h and followed by the 0.1% H_2O_2 treatment for 4h, decline in the activity of GST was observed. This decline in the activity of GST might be due to decreased levels of lipid peroxides formed for similar conditions as indicated by level of MDA formation (Table 3). As GSH is one of the co factors, decline in the activity of GST resulted in the increase GSH levels in the cells. For the similar conditions significant increase in the cell viability was observed. The decline in activity of LDH suggests reduced levels of oxidative stress experience by cells in comparison to the positive control (Rouach et al., 1997; Ramteke et al., 2007).

When the lymphocytes were pre-treated with the root extracts for 1h and followed by the treatment of 1% H_2O_2 for 4 h, decrease in the activity GST was found. As expected GSH levels increased and this increase was highly significant in comparison to the positive control at higher concentration. This might have results 200 fold increased in the cell viability up to 100 µg/ml of root extract treatment and thereafter at 200 µg/ml it declined. This decline in the cell viability at higher concentration of root extracts treatment might be due to the higher levels lipid peroxides and LDH activity. This suggests the synergistic action of the higher concentrations of root extract with the 1% H_2O_2 that have resulted in the decline in the cell viability. The exact reason for this finding is not clear from the present findings and needs further investigations (Parraga et al*.,* 2003; Dash et al., 2008).

From the present finding it could be concluded the root extracts of *Phlogacanthus tubiflorus* is rich in active principles with antioxidant properties and that might be responsible for the protective role against oxidative stress induced by H_2O_2 in the lymphocytes. GST and Glutathione have been implicated in the development of resistance towards chemotherapeutic agents. The therapeutic applications of the root extract of *Phlogacanthus tubiflorus* as modulators of GST and Glutathione needs further investigations in mammalian system.

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