

Induction of cell death through alteration of antioxidant activity in HeLa cervical cancer cells by *Xanthium strumarium* L. extract.

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Abstract: *Xanthium strumarium*, is a coarse unarmed annual herb with alternate lobed leaves, bur-like flower-heads, and small oblong fruits covered with curved hooks. The plant is reported to have diaphoretic, diuretic, sudorific, CNS depressant and stypic properties. Decoction of the plant is used in urinary and renal complaints, gleet, leucorrhoea and menorrhagia. In the present study, we used hydro-alcoholic extract of the plant against HeLa cell line to check the efficacy. HeLa cells were treated with the extract of *Xanthium strumarium* with the concentrations 12.5, 25 and 50 µg/ml at 24, 48 and 72 hours. Treated as well as untreated cells were analyzed for Lipid Peroxidation, as well as antioxidant defense enzymes, namely: Catalase, Superoxide Dismutase (SOD), Reduced Glutathione (GSH), Glutathione S-Transferase (GST), Glutathione Peroxidase (GPx) and Glutathione Reductase. The inhibition of antioxidants Glutathione, SOD and Catalase and increased levels of Lipid Peroxidation were observed in a concentration and time dependant manner. Together, our results suggest that the extract of *Xanthium strumarium* induces HeLa cell death by altering the antioxidant levels.

Key words: *Xanthium strumarium* · HeLa cell line · Antioxidative · LPO · SOD · Catalase · GSH · GST · GPx · GR

I. Introduction

Cancer refers to a diversity of diseases characterized by the uncontrolled proliferation of cells into a different form against the normal complement of the organism [1]. The continuous proliferation of cancer cells develops into tumour tissues and may spread across to other organs via circulatory systems resulting in metastasis. Orthodox cancer treatments include chemotherapy, radiotherapy, and surgery [2]. More often various types of treatment are combined to get the best possible result. The main mechanism by which these treatments act against various kinds of cancer is inhibition of growth proliferation and induction of apoptosis. Mechanism of Apoptosis can occur through two main pathways: death receptor- or mitochondria-mediated pathways.

The extrinsic pathway is triggered by extrinsic signals, such as growth factors, cytokines, extracellular hormones or components of the tumour necrosis factor family (TNF), which bind to death receptors on the plasma membrane. The intrinsic mitochondrial apoptotic pathway involves the mitochondria-to cytosol release of pro-apoptotic proteins. Different apoptotic stimuli, such as reactive oxygen and nitrogen species (ROS/RNS) and mitochondrial DNA damage, can mediate mitochondrial outer membrane permeabilization and the release of mitochondrial proapoptotic factors. Reactive oxygen species play a major role in signaling pathway for intrinsic mitochondrial apoptotic pathway. Many known drugs like Buthionine sulfoximine (BSO) and Verampil are known to act through this mechanism [3-4]. When we talk about plant components, chemopreventive activity and detoxification of carcinogens are the qualities of these components due to their antioxidant activities. Paradoxically, the pro-oxidant activity of phytochemicals may also contribute to their anticancer effects. Evidences suggest that certain polyphenolic compounds, certain Flavanoids, certain Glycosides, Phytosterols and Terpenoids have shown pro-oxidant effects generating ROS which in turn triggered the apoptotic pathway [5-10].

Xanthium strumarium is a well known herb which has been used since ages as a cure for various ailments like leucoderma, biliousness, epilepsy, salivation, fever and poisonous bites of insects [11]. The active components such as two sesquiterpene lactone glycosides and three kaurene glycosides isolated from *Xanthium* species are 3',4' didesulphated carboxyatractyloside and 3',4'-didesulphatedatractyloside [12], xanthanol, isoxanthanol and their C-4 epimers [13] have been reported. Xanthiazone and Caffeic acid have also been found from *Xanthium strumarium* [11]. In our previous studies we have shown that the *Xanthium strumarium* extract exhibits antiproliferative activity and also causes DNA damage leading to the possibility of apoptosis induction. [14]. Here we have probed further to support our studies in terms of mechanism of action and ROS generation, by the extract on HeLa cells.

II. Materials And Methods

2.1. Plant extract

Fruits of *Xanthium* were collected and authenticated by Botany Department, Gujarat University (Certificate No. GU/Bot/2013 Dated. 25/10/2013). Fruits were washed and shade dried under ambient temperature. After dried, the plants were sliced and ground using a steel blender. 50 g defatted (by hexane extraction) plant material was capsulated in filter paper and kept in the thimble, 500 ml solvent (water : methanol) (70 : 30) was added into the flask and continuous extraction was carried out in the soxhlet apparatus for 72 hours at 60-70° C till the colour in the siphon became colourless. The extract was dried at 60° C under fumehood till all the solvent got evaporated. 2-5% yield was obtained. The yield collected after drying was weighed and stored at 4°C until further use in sterile containers, as the hydroalcoholic extract.

The plant extracts for dosing were prepared with the concentrations 1 mg/ml in the Culture media with 5% DMSO and used further with various dilutions. The dose concentrations considered for the study were 12.5, 25 and 50 µg/ml and the tests were carried out after 24, 48 and 72 hours of the incubation after treatment.

2.2. Cancer cell culture

For cancer cell culture, HeLa cervix cell line ATCC®CCL-2™ was obtained from National Centre for Cell Science (NCCS), Pune. Cells were cultured in Minimum essential medium (MEM) with 10% fetal bovine serum and antibiotic antimycotic solution. All these supplements were procured from Hi-Media Laboratories. Cell cultures were maintained in a CO₂ incubator at 37°C. All the assays were carried out in the growth phase of the cells. For all the assays a definite number of cells i.e. 2 x 10⁶/ ml were used.

2.3. Reduced Glutathione (GSH):

The concentration of glutathione was estimated by the method of Ellman (1951)[15]. Cells were homogenized in phosphate buffer and TCA. After centrifugation, supernatant was taken to which of Ellman's reagent and phosphate buffer was added, while blank was run without homogenate. Mixture was incubated at 37° C for 30 minutes and absorbance was measured at 412nm. GSH Standards were treated in a similar way and the colour developed was read at 412 nm to make a linear plot. Absorbances of unknown samples were plotted against concentration with respect to standards.

Three independent assays were done and Glutathione levels were expressed as nmoles/2 x 10⁶ cells.

2.4. Glutathione reductase (GR)

Glutathione reductase activity was measured by the modified method of Carlberg and Mannervick (1985)[16]. Cells were homogenized in a known volume of potassium phosphate buffer (pH. 7.6) and centrifuged. To the assay mixture containing phosphate buffer, NADPH, EDTA, glutathione oxidized, enzyme source was added to initiate the reaction. The decrease in absorbance at 340 nm was followed for 3 minutes at exactly 1-minute interval. Blank tubes were run replacing sample supernatant (enzyme) with buffer. The difference in the absorbance was obtained by subtracting the blank readings from the sample readings.

The activity of Glutathione reductase was calculated as follows:

$$\text{Glutathione reductase activity} = \frac{\Delta A / \text{min} \times \text{volume of assay}}{6.22 \times 10^6 \times \text{volume of enzyme source} \times \text{mg protein}}$$

Three independent assays were done and the activity of glutathione reductase was expressed as nmol NADPH oxidized/ min/mg protein.

2.5. Glutathione Peroxidase (GPx)

Activity of GPx was assayed by the method of Rotruck *et al.*, (1973)[17]. Cells were homogenized in Tris-HCl buffer, pH 7.0. Aliquots were added in all test tubes except blank. Blank was run with equal volume of Tris- HCl buffer. Further, sodium azide, glutathione and hydrogen peroxide were added to reaction mixture and the contents were incubated at 37° C for 10 min. The reaction was arrested by 10% TCA and centrifuged. Supernatant was assayed for Glutathione content by using Ellman's reagent as mentioned in GSH assay.

Three independent assays were done and GPx activity was estimated as –moles GSH consumed /2 x 10⁶ cells.

2.6. Glutathione S-Transferase (GST) Activity:

The GST activity was determined spectrophotometrically at 37°C according to the procedure of Habig *et al.*, 1974 [18]. The reaction mixture containing phosphate buffer (pH 6.5) and CDNB(1-Chloro-2,4-

dinitrobenzene) was preincubated at 37°C for 5 minutes, and the reaction was initiated by the addition of supernatant and the absorbance recorded for 5 minutes at 340 nm in a UV-Visible double-beam spectrophotometer. Reaction mixture sans enzyme was used as blank.

$$\text{Units / ml enzyme} = \frac{\Delta A_{340} / \text{min test} - \Delta A_{340}}{\text{min blank}} \times 3 \times \text{df} \times 9.6 \times 0.10$$

3.0 = Total volume

df = dilution factor

9.6 = millimolar extinction coefficient of Glutathione -1-chloro-2,4- Dinitrobenzene conjugate at 340 nm

0.10 = volume of enzyme source

Three independent assays were done and GST activity has been expressed as units/mg protein.

2.7. Superoxide Dismutase (SOD) Activity:

The activity of superoxide dismutase was assayed by the modified method of Kakkar *et al.*, (1984) [19]. Cells were washed with PBS and then homogenized in cold normal saline. The tubes were then centrifuged. The supernatant was discarded and to the pellet 0.01% digitonin was added to yield the enzyme extract. After 15 minutes of incubation, the tubes were centrifuged and the supernatant was used as sample. In the assay system, the control consisted of phosphate buffer, freshly prepared phenazine methosulphate nitroblue tetrazolium (NBT) and fresh NADH. To the sample system 0.2 ml of enzyme was added prior to the addition of NADH in a total of 3 ml of assay system. The reaction was stopped by the addition of acetic acid exactly 90 seconds after the addition of NADH. n- Butanol was added to the tubes and shaken vigorously to extract the formazan. Then the tubes were centrifuged for 10 minutes and supernatant was used for the measurement of optical density at 560 nm against butanol on a Visible Spectrophotometer.

$$\text{SOD Activity} = \frac{\text{Absorbance of control}}{\text{Absorbance of sample}} \times \text{accuracy factor} \times \frac{\text{dilution}}{\text{standard enzyme units}}$$

Where, accuracy factor = 1; dilution = 2.5 ; standard enzyme unit = 3.0

Enzyme activity = units/ mg protein

Three independent assays were done and the activity was expressed as units SOD / mg protein.

2.8. Catalase Activity:

The Catalase activity was assayed by the modified method of Sinha (1972) [20]. Briefly, the incubation mixture had volume containing dilute homogenate, phosphate buffer and distilled water to which H₂O₂ solution was added. After incubating for 1 minute at 37° C the reaction was stopped by the addition of potassium dichromate acetic acid reagent. The samples were kept in boiling water bath for 15 minutes, finally cooled and the absorbance was measured at 570 nm against control.

$$\text{Catalase Activity} = \frac{\text{Absorbance of sample} \times \text{standard concentration} (\mu\text{mol})}{\text{enzyme (ml)} \text{ Std. Absorbance} \times \text{Protein (mg / ml)}}$$

Three independent assays were done and the activity was measured as μ moles H₂O₂ consumed / min/ mg protein

2.9. Lipid Peroxidation (LPO)-Thiobarbituric Acid Reactive Species Assay (TBARS):

The thiobarbituric acid reactive species (TBARS) level in cells was determined by the method of Okhawa *et al.*, (1979) [21]. Cells were washed several times with phosphate buffered saline (PBS) (pH. 7.4) and were homogenized to prepare cell homogenate. Homogenate was added to the tubes containing sodium dodecyl sulphate (SDS), acetic acid solution and 1% Thiobarbituric acid (TBA) solution. The blank was prepared for each sample by substituting TBA solution with distilled water. The solution was mixed and heated in a water bath at 95 ° C for 60 minutes. The tubes were then immediately cooled and aliquot was transferred to a centrifuge tube to which an equal volume of 10 % TCA was added. The solution was mixed and centrifuged at for 15 minutes. The aliquot of the resulting supernatant fraction was read against blank on Systronics Digital Spectrophotometer 167 at 532 nm.

$$\text{Concentration of MDA} = \frac{\text{Absorbance of sample} \times \text{dilution factor} \times 10^9}{E \times 10^6 \text{ cells}}$$

Where dilution factor = 0.5

E= extinction co efficient for MDA (1.56 x 10⁵)

Three independent assays were done the results were expressed as nanomoles of MDA / 10^6 cells /60 minutes.

2.10. Statistical analysis

For each parameter was performed in triplicate and the results were expressed as Mean \pm Standard Error (S.E.M). the data was the statistically analysed by Students 't' test. In all the parameters, resultant effect of treated cells were compared with untreated control cells.

III. Results:

3.1. Reduced Glutathione (GSH)

An insignificant decrease was observed in reduced Glutathione level at low dose 12.5 μ g/ml for 24 and 48 hours. There was a significant decrease in the levels of GSH at higher concentrations. The decrease observed was time and concentration dependant. Significant changes in the GSH levels were not observed for the control cells at various time intervals (Fig. 1).

3.2. Glutathione Reductase (GR) Activity

A gradual decrease in the GR activity was observed with increase in time and concentration in the treated cells, whereas there was not much difference in the activity of untreated control cells (Fig. 2). A highly significant reduction in the activity of GR was noticed in the extract treated cells (50 μ g/ml) at 72 hours.

3.3. Glutathione Peroxidase (GPx) activity

A highly significant decrease in the GPx activity was observed with increase in time and concentration of the extracts in the treated cells, whereas there was not much difference was observed in the activity of untreated control cells (Fig. 3).

3.4. Glutathione S-Transferase (GST) Activity

The GST activity of control and treated HeLa cells are given in (Fig. 4). A significant decrease in the GST activity was observed with increase in time and concentration of the extracts in the treated cells, whereas there was not much difference was observed in the activity of untreated control cells.

3.5. Superoxide Dismutase (SOD) Activity

The results showed that there was reduction in SOD activity which was time and concentration dependant. When compared with the control, highly significant decrease in the activity of SOD was observed with 50 μ g/ml concentration for 72 hours duration. The results of the SOD activity of the control and different plant extracts are shown in Fig. 5.

3.6. Catalase Activity

The results of the Catalase activity of the control and treated HeLa cells are shown in Fig. 6. There was a significant decrease in the activity which was again time and concentration dependant as compared with the untreated cells. At the highest concentration and time *Xanthium* extract had shown a decrease of 65% activity of Catalase when compared with control.

3.7. Lipid peroxidation

The levels of alteration of LPO are depicted in Fig. 7. The results showed that there was increase in Lipid Peroxidation in the treated cells as compared to the untreated control cells which was time and concentration dependant. When compared with the control, highly significant increase in the LPO levels was observed at highest concentration for 72 hours duration.

IV. Discussion

Over the past decade, herbal medicine has become a topic of global importance, making an impact on both world health and international trade. Medicinal plants continue to play a central role in the healthcare system of large proportions of the world's population [22].

Xanthium strumarium extract in our previous studies showed selective *in vitro* cytotoxicity, and antiproliferative activity against HeLa cancer cell line. Our results further showed that apoptosis might be the cause of cell death inhibiting growth proliferation. For the detection of apoptosis we studied the DNA fragmentation parameters. Our Studies showed that the extracts induced apoptosis in this model of cancer cell line in a time and concentration-dependent fashion [14].

It could be established from the previous studies that the extracts induce DNA breaks and apoptosis might be the cause for the cell death. The results obtained in this study showed that there was decrease in

Reduced Glutathione levels, Glutathione Peroxidase and reductase activity and Glutathione S- Transferase activity. SOD and Catalase levels have also been observed to have been decreased along with increase in Lipid Peroxidation. All these results indicate that there was ROS generation after the cells were treated with the extracts.

Cancer cells have evolved mechanisms to protect themselves from intrinsic oxidative stress and have developed a sophisticated adaptation system that essentially involves the rearrangement of the antioxidant functions that is, increased activity of the antioxidant enzymes and the up regulation of pro-survival molecules [23]. Cells have evolved several antioxidant defenses, including repair and detoxifying enzymes, and small scavenger molecules, such as glutathione. The cellular redox balance is maintained by a powerful antioxidant system that “neutralizes” ROS. This intracellular ROS-scavenging system includes superoxide dismutases (SOD), glutathione peroxidase (GPx), glutathione S-transferases (GST) and Catalases. The strategy of depleting GSH and GSH-related detoxification pathways is aimed at sensitizing cancer cells to chemotherapy, the so-called chemosensitization [24]. Increased concentration of GSH in the tumor cells have been reported to make the tumor refractory to treatment, while depletion of glutathione has been reported to enhance the cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of the calcium homeostasis and eventually loss of cell viability [25-26]. In this context, it has been reported that GSH and GSH enzyme-linked system may be a determining factor for the sensitivity of some tumors to various chemotherapeutic agents. Treatments with the extracts in the present study lead to depleted levels of GSH and decrease in GST activity lead us to believe that there is decrease in the rate of conjugation and detoxification leading to chemosensitization of the cell rendering the extracts effective in inducing apoptosis and eventually causing cell death.

In mitochondria, superoxide anion ($O_2^{\cdot-}$) can be dismutated to hydrogen peroxide (H_2O_2) by two enzymes, namely copper-zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD), that are present in the mitochondrial matrix and in the intermembrane space, respectively. Once generated, H_2O_2 can be quenched by GPx in mitochondria, or by catalase in the cytosol [27]. Hence, Together with SOD, Catalase and Glutathione Peroxidase also have a central role in the protection of cancer cells against intrinsic oxidative stress. For this reason SOD, Catalase and GPx inhibitors can contribute to the resensitization of cancer cells to ROS-induced apoptosis [28]. The results indicated that the dose and time dependant decrease in SOD, Catalase and GPx activities give rise to the possibility that the protective mechanism of the cells was rendered ineffective and the cancer cells probably are getting resensitized, by the effect of the extracts, which leads to further mechanisms triggering apoptotic cell death.

Although cancer cells have developed adaptive mechanisms to minimize the effects of oxidative damage, excessive ROS levels can disrupt redox homeostasis, and hence affect death or survival fate, either by irreversibly damaging cellular macromolecules, including DNA, carbohydrates, protein, and lipids [29]. We found that the extracts increased the lipid per-oxidation with a simultaneous decrease in cell proliferation in cervical cancer cell line which might be the result of decreased Gpx activity leading to generation of oxidative stress mediated cell death.

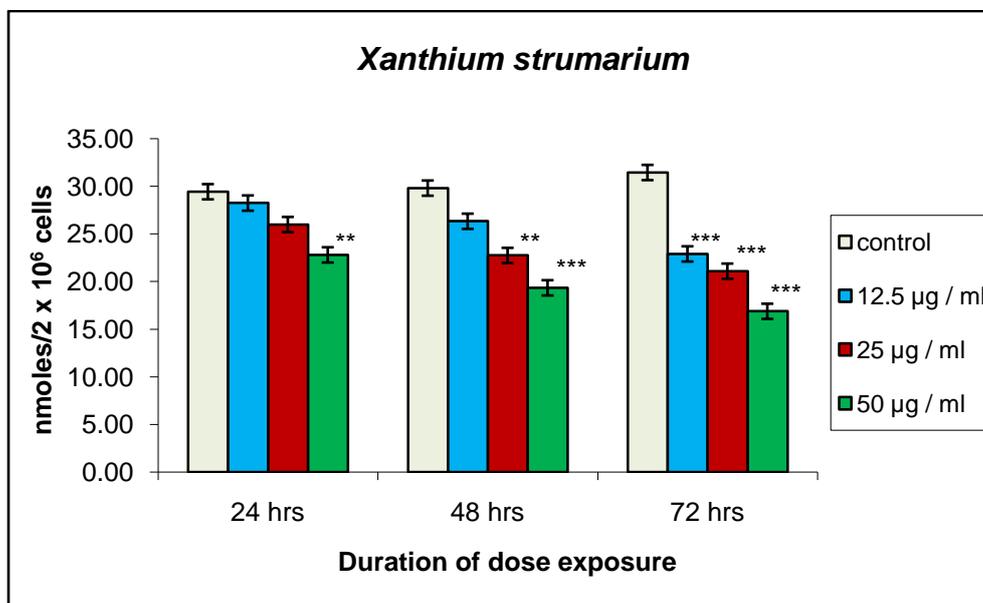
One of the main features of cancer cells, when compared to the normal ones, is a persistent pro-oxidative state that can lead to intrinsic oxidative stress [30-31] and therefore, it has become very challenging to develop new therapeutic strategies due to the persistent pro oxidative state as well as their multiple adaptations. Hence, there is a need to develop new ways to specifically target redox alterations, in order to ensure a good therapeutic selectivity. Because the overall redox homeostasis is maintained by the balance between ROS generation and elimination, exogenous compounds that increase ROS generation, or inhibit ROS elimination, can favor the accumulation of ROS in cancer cells, and hence induce cell damage or even cell death [32]. Several agents have been identified that promote ROS generation, *i.e.*, mitochondrial electron transport chain modulators (e.g., arsenic trioxide), redox-cycling compounds (e.g., motexafin gadolinium), or that disrupt antioxidant defenses, *i.e.*, GSH depleting agents (e.g., buthionine sulphoximine), and inhibitors of SOD (e.g., 2-methoxyestradiol), and catalase (e.g., 3-amino-1,2,4-triazole) [32-34]. As messengers of signal transduction, ROS target key signaling molecules, such as mitogen-activated protein kinases (MAPK), protein phosphatases and transcription factors [35-38]. Hence, ROS in this manner is responsible for apoptotic induction in a cell.

A known example of plant component acting through this mechanism is Quercetin. In certain situations, Quercetin can generate enough ROS to cause free radical-induced apoptosis through, at least, the ROS/AMPK α 1/ASK1/p38 and the AMPK α 1/COX2 signaling pathways [39]. Several dietary polyphenols such as resveratrol and caffeic acid have been shown to cause cellular DNA damage through mobilization of endogenous copper ions, possibly chromatin-bound copper, leading to the production of ROS [40-44]. Caffeic acid is one of the active component found in *Xanthium strumarium* plant [11]. It is possible that the alteration in the levels of the antioxidants in the cells might be due to the action of this component. Though further studies are required on this line associating the active component and the ROS generation, it can be said that the components present in the extract alone or in combination are responsible for the alteration in antioxidant levels

in HeLa cells, which in turn might have triggered the apoptotic mechanism resulting in DNA fragmentation and eventually cell death.

V. Figures

Fig. 1. Showing the levels of Reduced Glutathione (GSH) in HeLa cells treated with *Xanthium strumarium* extract compared with untreated control cells.

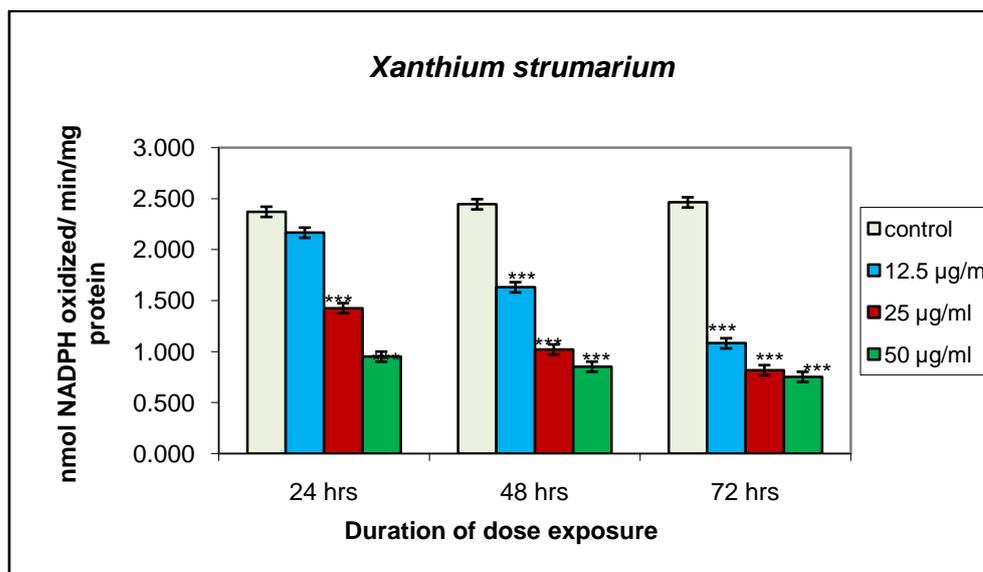


Values are mean ± S.E.M for three individual experiments

Values are nmole GSH / 2 x 10⁶ cells.

ns = non-significant, **= p < 0.005 ***= p < 0.001

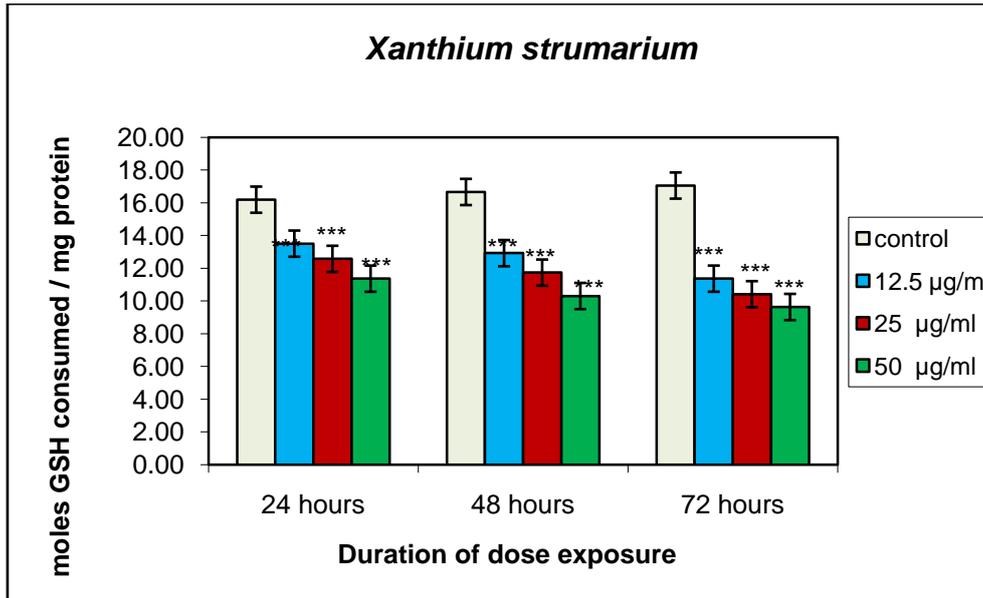
Fig. 2. Showing alterations in Glutathione Reductase (GR) activity after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.



Values are mean ± S.E.M for three individual experiments

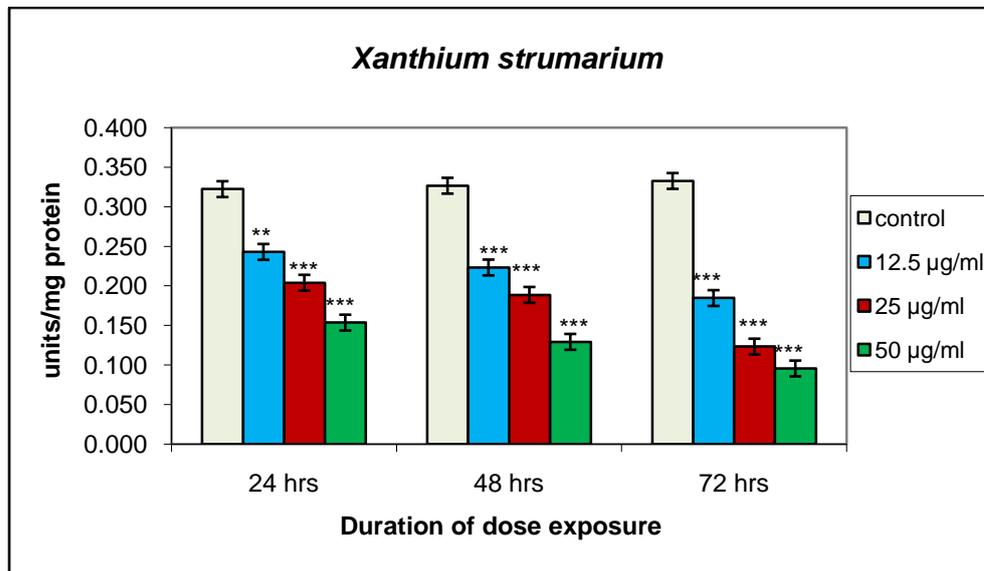
Values are nmole NADPH oxidized/ min / mg protein. ns = non-significant, ***= p < 0.001

Fig. 3. Showing alterations in Glutathione Peroxidase (GPx) activity after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.



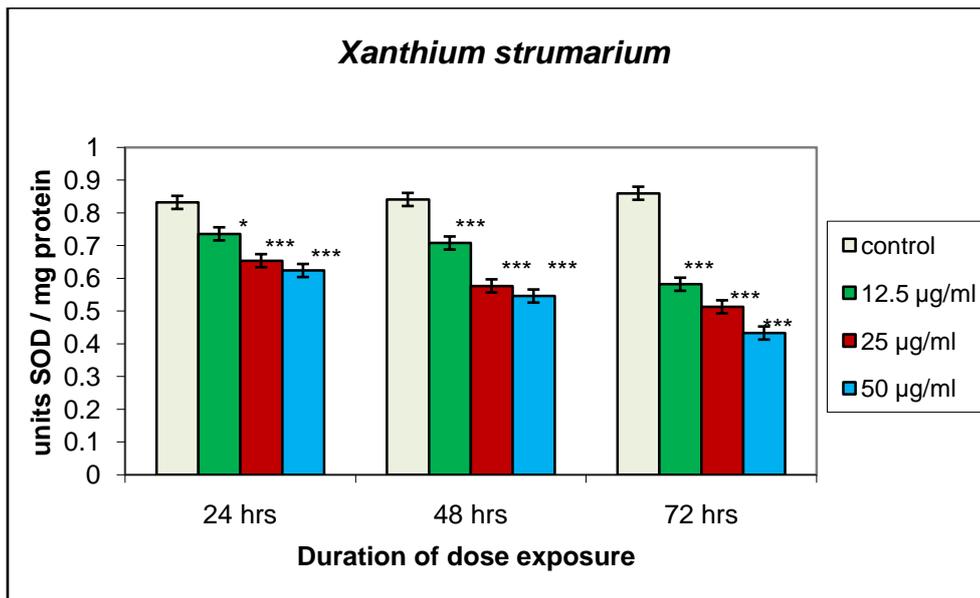
Values are mean ± S.E.M for three individual experiments
 Values are moles GSH consumed / mg protein.
 ***= p < 0.001

Fig. 4. Showing alterations in Glutathione S-Transferase (GST) activity after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.



Values are mean ± S.E.M for three individual experiments
 Values are units / mg protein. **= p < 0.005, ***= p < 0.001

Fig. 5. Showing alterations in Superoxide Dismutase (SOD) activity after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.

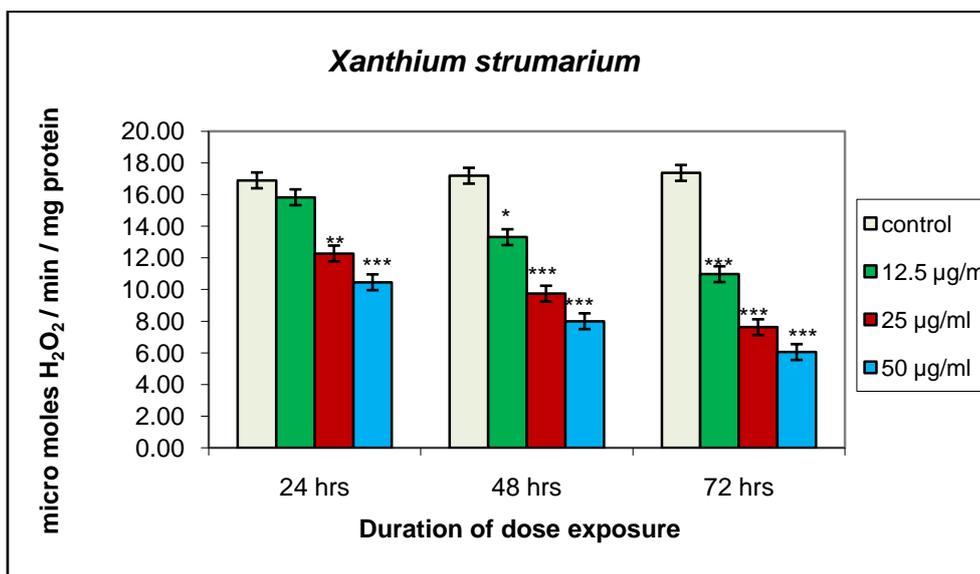


Values are mean ± S.E.M for three individual experiments

Values are units SOD / mg protein.

*= p < 0.01, ***= p < 0.001

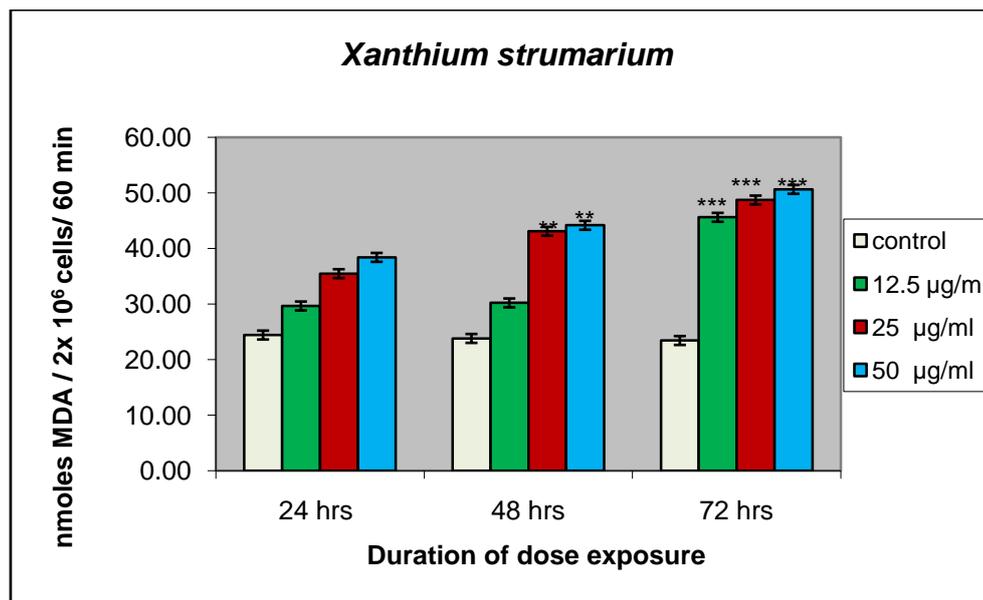
Fig. 6. Showing alterations in Catalase activity after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.



Values are mean ± S.E.M for three individual experiments

Values are µ moles H₂O₂ consumed / min/ mg protein. ns = non-significant, *= p < 0.01, **= p < 0.005, ***= p < 0.001

Fig. 7. Showing alterations in Lipid Peroxidation activity after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.



Values are mean \pm S.E.M for three individual experiments

Values are nmoles of MDA / 2×10^6 cells / 60 minutes.

ns = non-significant , ** = $p < 0.005$, *** = $p < 0.001$

VI. CONCLUSION

There is alteration in levels of the antioxidants after the treatment of HeLa cells with the *Xanthium strumarium* extract due to ROS generation. ROS might be acting as specific second messengers in the signal transduction pathway eventually resulting in apoptotic induction and causing cell death. Though role of *Xanthium* extract remains to be explored further, it can be suggested that *Xanthium strumarium* can be considered as a possible therapeutic agent against human carcinomas.

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