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## Regulation of Acrylamide Induced $\mu$ -Class Glutathione-S-Transferases in Mice Testis by the Treatment of *Hybanthus Enneaspermus* Active Principles

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### ABSTRACT

The present study was aimed to analyse the effect of acrylamide and *Hybanthus enneaspermus* leaf extract active principles on mice testis glutathione-s-transferases (GST; EC 2.5.1.18). These enzymes play a role in biotransformation of electrophilic compounds that cause damage to cells by conjugating with the substrate glutathione. *Hybanthus enneaspermus*, a spade flower, is an erect shrub of violaceae family, having free radical scavenging activity. Acrylamide is a known neurotoxicant that cause damage to almost all cells including liver, testis, brain and kidney. The GSTs purified from mice testis using glutathionyl linked agarose affinity chromatography were analyzed by using SDS-PAGE and were resolved into four sub units i.e. Yc, Yb, Y $\beta$  & Y $\delta$ . Also these subunits expression were confirmed by western blot analysis. During experimentation to analyze the effect of *Hybanthus enneaspermus* active principle (HE) mice were subjected to both acrylamide (AC) and also mixture of HE and AC. This exposure significantly altered the specific activity of mice GSTs in testis. Polyclonal antibodies produced against purified GSTs of mice testis on immunoblot analysis showed significant increase of  $\mu$ -class GSTs (Yb & Y $\beta$ ) based on dose and time dependent manner. Therefore the present research of *Hybanthus enneaspermus* treatment on mice testis showed that, regulation of synthesis of  $\mu$ -GSTs was depending on the dose of acrylamide concentration and also the active principles of HE. Hence it is proposed that  $\mu$ -GSTs may be used as tumour markers for testis carcinoma, since their production is variable due to the increased dose concentration of synthetic chemical acrylamide and its regulation by plant product, HE.

**Keywords:** Glutathione-S-transferases, testis, Acrylamide, Western blotting, *Hybanthus enneaspermus* active principle.

### INTRODUCTION

The Glutathione-S-transferases (GST, EC 2.5.1.18) are multi protein enzymes that participate in cellular detoxification of endogenous as well as foreign electrophilic compounds, and are evolved to protect cells against reactive oxygen species (ROS) by conjugating the reactive molecules to an electrophile scavenging tripeptide, glutathione (GSH,  $\gamma$ -glu-cys-gly). However, Gallagher *et al.*, (1996) have reported that GST detoxifies a number of environmental carcinogens and epoxide intermediates due to its multifunctional properties.

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Thus, the GST assay was suggested as a useful tool for biomonitoring oxidative stress (Di Giulio *et al.* 1993). The cells are protected from reactive epoxides and oxygen species by GSTs (Yuen *et al.*, 2001; Raveendra *et al.*, 2008).

Chemical stress on the body may actually cause production of ROS which results in oxidative stress on cells, which in turn activates GST activity (Feng *et al.*, 2001). The role of GSTs as an antioxidant enzyme can reduce the lipid peroxides to form their respective alcohols by using GSH and thereby prevent oxidative damage to the cells (Barata *et al.*, 2005). In rats, an antioxidant responsive element (ARE) has been identified in the 5' flanking region of the GST Ya subunit gene (Rushmore *et al.*, 1991). The ARE is a *cis*-acting regulatory element that is responsive to oxidative stress and protect the cells from it (Rushmore *et al.*, 1991; Feng *et al.*, 2001).

The GST proteins are versatile, multifunctional and exist in more than two subunits. Each subunit of GST has a characteristic enzyme activity, which is expressed independently of the other subunit (Ostlund Farrants *et al.*, 1987). The GSTs in addition to their enzymatic activities, bind with high affinity to a variety of hydrophobic compounds such as heme, bilirubin, hormones and drugs, which suggests that they may serve as intracellular carrier proteins for the transport and biotransformation of various ligands. A marked increase in GST activity has been observed in tumour cells resistant to anticancer drugs (Daniel, 1993) hence GSTs can protect organisms from toxic chemicals once they are ingested or absorbed from the surrounding environment (Clark, 1989). GSTs are involved in the first enzymatic step in formation of mercapturic acids (N-acetyl L-cysteinyl S-conjugates), by converting toxic compounds into a water soluble, nonreactive conjugates which may easily be excreted through kidney (Clark, 1989). The enzymes do this by catalyzing the conjugation of GSH to the xenobiotics and then in turn make the xenobiotics more hydrophilic for easy excretion by the organism using mercapturic acid pathway (Vidal *et al.*, 2002). Acrylamide (AC), an odourless, white, crystalline solid, is a multisite carcinogen. It induces cancer to the testes due to male reproductive toxicity and affecting male fertility and heritable genetic mutations in male mice and rats (CERHR, 2004a&b). In this context, the present study was aimed to reveal the effect of selected chemical toxicant, acrylamide, on the induction of glutathione S-transferases and investigate the sustained induction of GSTs in testis of mice with selected dose and time intervals and also under the influence of active principle of *H. enneaspermus*. The plant *H. enneaspermus*, is considered to be potential inducer for the production of sperm in males due to the influence of the active principle on testis was studied below and discussed its effect as described.

## MATERIALS AND METHODS

### Plant material

*Hybanthus enneaspermus* (Ratna pursha) plants, a spade flower were collected from the fields of SVU campus, Tirupati, Chittoor District, A. P, and mice were purchased from Sri Venkateswara enterprises, Bangalore.

### Preparation and analysis of plant extract on HPLC & TLC

Fresh plant leaves collected fields were washed thoroughly using tap water and 100 gm of leaves were ground and mixed with 200 ml of absolute ethanol and allowed to settle for overnight at 4°C. The resulted ethanolic extract was decanted and lyophilized at 0°C to make powder and the collected powder was stored at 4°C for further investigations. This powder was considered as *Hybanthus enneaspermus* active principles extract (HEAPE) (Haseena Bhanu *et al.*, 2011).

The HEAPE, 20 mg by weight/ml, was dissolved in one of litre methanol and filtered through sintered microporous funnel. This preparation was allowed to dissolve for 24 hours and the dissolved sample (upper layer) was collected and evaporated using soxhlet evaporation. The sample was almost dried and diluted to one to five ml in methanol, and allowed for injection to HPLC reverse phase C<sub>18</sub> chromatography column (30cm x 0.5cm). The injected sample fractions were separated using the mobile phase of methanol and water (70:30 v/v), and the peak fractions collected were further analysed for their biological activity.

Thin layer chromatography was also carried out on Merck TLC plates (silica gel coated) for, separation of active compounds of collected fractions using HPLC in a solvent saturated chamber with suitable solvent system i.e., ethanol: chloroform (7:3). The separated molecules on TLC were visualized under ultraviolet light after exposure to iodine vapours.

### Phytochemical screening

Phytochemical screening was performed using standard procedures (Sofowora, 1993; Trease and Evans, 1989).

### Acrylamide treatment

Acrylamide, a highly water-soluble  $\alpha$ ,  $\beta$ -unsaturated amide, reacts with nucleophilic sites in macromolecules in Michael-type additions (Calleman, 1996; Segerbäck *et al.*, 1995). Monomeric AC readily participates in radical-initiated polymerization reactions, whose products form the basis of most of its industrial applications (Calleman, 1996). A study was made on the mice testis tissue for specific GST expression after 24 hours exposure of mice to acrylamide concentration of 1mg to 4mg per kg body weight of animal from day 1 to 4 and determined the activities and expression of new GST proteins which are involved in the detoxification process.

### Plant extracts treatment

The male mice weighing about 50 gm (3 months old) were used for treatment studies. The mice were treated simultaneously with i.p. administration of HEAPE and acrylamide with various intervals. The mice were maintained by feeding the standard pellet and maintained *ad libitum* with water and sacrificed after the last dose of treatment. Control vehicles were also maintained by giving HEAPE.

The mice were treated with i.p. administration of mixture of HEAPE and acrylamide for four days with an interval hours of 24, 48 and 72 to a total concentration of 4 mg at the rate of 1mg per 0.5ml of each dose and the mice were sacrificed after 24 hours

interval of the each dose. The testis of the control and treated mice were collected after decapitation and stored at -20°C until further use.

### Processing of tissue for GST Activity

Normal and treated testis were thawed separately, minced with scissors and homogenized in 50 mM Tris-HCl buffer, pH 8.0, containing 0.25M sucrose and 1mM PMSF using a glass homogenizer. Homogenization was done by Potter-Elvehjem homogenizer in an ice jacket and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheese cloth to remove fat and debris, and was centrifuged at 10,000 x g on high speed refrigerated centrifuge (Remi) for 30 min. The resulting supernatant was used as the enzyme source. All the purification procedures were conducted at 4°C unless otherwise stated.

Mice testicular GSTs were purified to electrophoretic homogeneity by GSH-Affinity chromatography (Ruxana Begum and Thyagaraju, 2010). SDS-PAGE of affinity purified cytosolic testis GSTs resolved into four bands with relative molecular weights of 27.5(Yc), 26.3(Yb), 26.0(Yβ) and 24.8(Yδ) in kDa.

Polyclonal antibodies were raised in rabbits against affinity purified testis GSTs and the expression of the GSTs were observed by using Western-blot analysis with class specific polyclonal antibodies (testis) with various concentrations of acrylamide treated testis cytosols. The quantification of subunits in both control and treated tissue of testis on immunochemical analysis, and also by enzymatic assays in a dose-dependent manner was carried out using battery of substrates as described by Thyagaraju *et al.*, (1996).

## RESULTS

The mice treated with various concentrations of acrylamide with an interval of 24 hours and GST and Gpx activities observed were tabulated for testis in Table-1. This data has revealed that α-class GSTs (Yc, Ya) and μ-class GSTs (Yb, Yβ) are expressed predominantly more in testis on AC treatments.

The increased levels of activity with EPNP, pNPA and pNBC indicated the presence of elevation of μ-GST in mice testis due to increased dose of acrylamide.

Similarly in the present study of acrylamide treatment mice showed the increased amounts GSH peroxidase activities. These studies indicate that the elevated levels of activity with CHP and H<sub>2</sub>O<sub>2</sub>. The increased level of activity on CHP and H<sub>2</sub>O<sub>2</sub> is an indication of α-class GSTs in mice testis. However the decreased level of activity using BSP indicated the decreased GST activity of π class upon acrylamide treatment (Table 1). The free radicals generated in the testis must have used of GSH as substrate to reduce the oxygenated molecules to convert them into hydrophilic compounds. Due to the available GSH absence in the mice GPx must have stopped in mice and the mice must have induced GST proteins to scavenge the molecules to protect various cell types of kidney, testis and other organs.

The effect of the mixture of acrylamide and HE is presented in Table 1, and figures 4 and 5. The effect of acrylamide was suppressed by the active principle of HE was known from this table. The activities of GSTs on all substrates showed less activity levels as compared to acrylamide alone. HE showed less GST activities to all substrates. Hence the molecules those are present in HE may serve as nucleophile to regulate the effect of acrylamide in testis of mice. The decreased GST activity levels indicated the normalization of system in testis by HE.

### *Hybanthus enneaspermus* active principle:

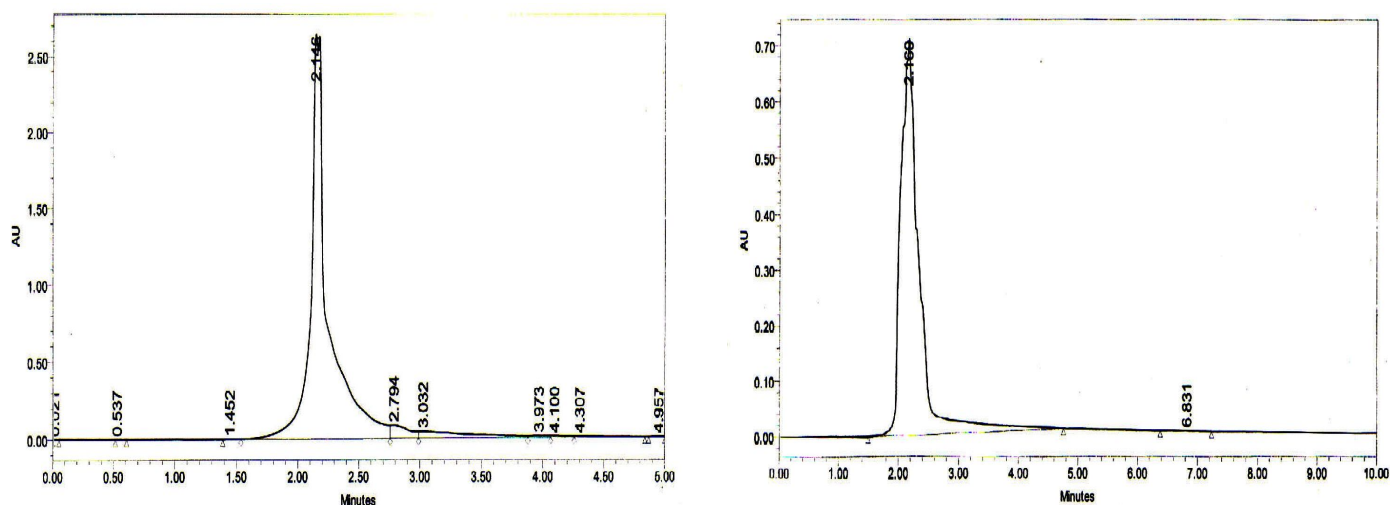
The *Hybanthus enneaspermus* active principle extract was isolated using Soxlet evaporation followed by HPLC using the C<sub>18</sub> reverse phase chromatography column. The HPLC profile was depicted in Figures-1 (A and B) and its spectral properties were showed in Figure-3.

Both figures on elution from the column showed a single sharp peak at retention time of about 2.146 to 2.169 min at the wavelength of 240nm. The HPLC isolated fraction an analysis of thin layer chromatography found to contain a single compound with a minor contaminant.

**Table. 1:** Table shows the effect of Acrylamide, Acrylamide and *Hybanthus enneaspermus* (24hours interval) on the levels of GSTs and GPx of mice testis with different substrates.

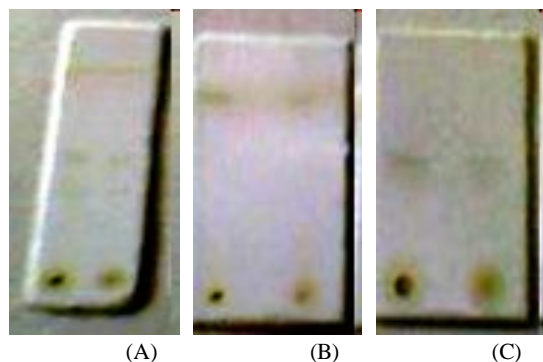
|         | CDNB        | EPNP      | pNPA        | pNBC        | BSP         | CHP         | H <sub>2</sub> O <sub>2</sub> |
|---------|-------------|-----------|-------------|-------------|-------------|-------------|-------------------------------|
| Control | 21.14 ± 3.1 | 0.58±0.50 | 0.072±0.008 | 0.428±0.015 | 0.090±0.006 | 0.625±0.018 | 0.303±0.030                   |
| 1mgAC   | 54.11±6.5   | 0.92±0.62 | 0.138±0.042 | 0.710±0.42  | 0.23±0.016  | 0.801±0.24  | 0.514±0.065                   |
| 2mgAC   | 63.24±6.8   | 1.23±0.82 | 0.161±0.056 | 0.938±0.55  | 0.46±0.025  | 1.240±0.28  | 0.828±0.090                   |
| 3mgAC   | 79.30±7.1   | 1.68±0.92 | 0.194±0.062 | 1.251±0.79  | 0.63±0.046  | 1.623±0.52  | 1.381±0.233                   |
| 4mgAC   | 81.00±7.2   | 1.69±0.97 | 0.199±0.068 | 1.301±0.82  | 0.78±0.053  | 1.623±0.52  | 1.381±0.233                   |
| HE      | 18.09±2.8   | 0.49±0.40 | 0.061±0.006 | 0.404±0.010 | 0.087±0.003 | 0.609±0.015 | 0.285±0.026                   |
| 1 mg AH | 33.09±4.6   | 0.79±0.7  | 0.099±0.016 | 0.489±0.018 | 0.19±0.007  | 0.768±0.021 | 0.398±0.039                   |
| 2 mg AH | 45.12±5.4   | 0.86±0.56 | 0.118±0.026 | 0.527±0.24  | 0.28±0.014  | 0.709±0.021 | 0.306±0.039                   |
| 3 mg AH | 46.19±5.46  | 0.90±0.61 | 0.126±0.032 | 0.536±0.34  | 0.32±0.018  | 0.692±0.022 | 0.265±0.031                   |
| 4 mg AH | 49.99±6.0   | 0.91±0.64 | 0.134±0.038 | 0.610±0.4   | 0.34±0.020  | 0.642±0.019 | 0.221±0.028                   |

NOTE: Values are average of three separate experiments of three samples. Mean ±SD significant. The GST activity values are expressed in males of GS – conjugate formed per min per mg protein and GPx activities are expressed as oxidation of GSH per min per mg of protein. (AH is Acrylamide + *Hybanthus enneaspermus* mixture)

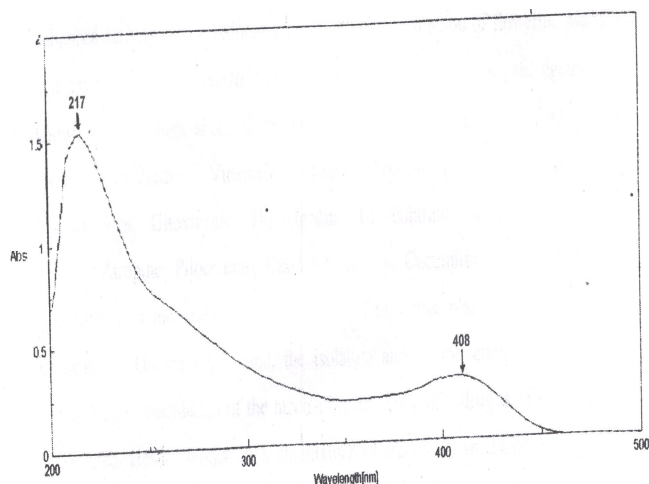


**Fig. 1:** HPLC Profile on the isolation active principle of *Hybanthus enneaspermus* leaf extract. (A) Active principle activity resolved into two peaks. (B) Active principle activity resolved into a single peak (pure compound)

The TLC isolated fractions on scanning  $\lambda$  maxima absorption peaks at 217nm and 408nm. This indicates that the compound have an aromatic molecule linked to alkyl carboxylic link. Therefore the compound isolated from HE was almost pure active principle used for its effect on mice testis GSTs and GST associated peroxidase enzymes.



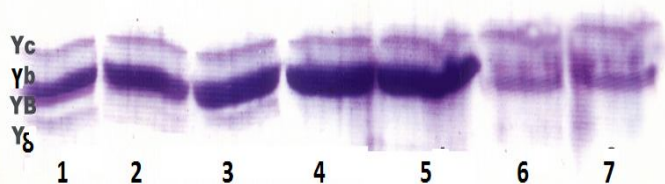
**Fig. 2 :** TLC analysis of the ethanolic extract of the purified sample of *H. enneaspermus*.



**Fig. 3:** Absorption spectrum of selected molecule of *H. enneaspermus* ethanolic extract.

## WESTERN BLOTTING

Antisera raised against affinity purified GSTs of mice testis on transblot analysis showed immunoprecipitin bands with both control and acrylamide treated mice testis tissue homogenates. Testis tissue homogenates were showed cross reactivity with GST proteins after transblot and immunoprecipitin analysis (**Figure-4**). The results of acrylamide treatment (**Figure-1** and **lanes 3-6**) showed the elevated expression of Yb subunit of  $\mu$ -class GST and two GSTs were almost disappeared on gel. The disappeared proteins are Y $\beta$  and Y $\delta$  protein of testis (**lanes 5-6**).



The gel lanes: Lanes: 1-5 AC+HE, Lane: 6 control, Lane: 7 HE

**Fig. 4:** Immunoblot analysis of mice testis of both control and acrylamide induced GSTs probed with GST specific antibodies (BCIP/NBT staining).

Further to observe the suppression of expression of  $\mu$ -class GSTs the effect of HEAPE was analysed as the mice exposed to acrylamide. The results are presented in figure. The lanes 1 to 4 of transblot on reaction with antibody of GST showed elevated expression of  $\mu$ -class GST and the lane 5 showed normalization of expression of GST upon HE treatment.

## HISTOPATHOLOGY

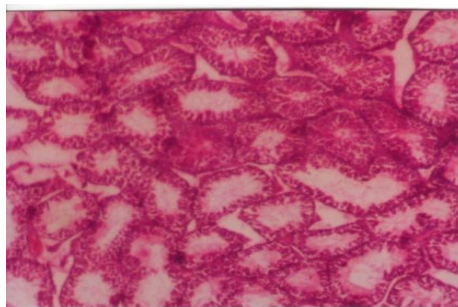
Histological changes of testis were observed under light microscope and the following results were presented from figure 5. The control mice testis showed numerous seminiferous tubules (ST) with a connective tissue as a boundary line and developing spermatids and developed spermatozoa (Fig. 5a).

Male mice treated with AC (1mg/100 gm body weight / 48 hours interval for three days). The administration of acrylamide

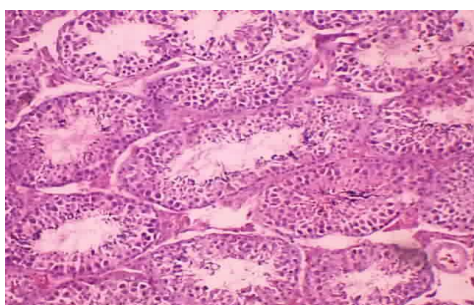
showed pathological symptoms in testis according to dose and time. Mice testis of 48 hours interval showed degenerative changes in the lumen and seminiferous tubules, necrotic spermatids and atrophied seminiferous tubules, extensive interstitial oedema and pyknotic nuclei, arrest of spermatogenesis and also degenerative changes in primary and secondary spermatogonia. Other symptoms included testicular atrophy and degeneration of

germinal epithelium. Arrest of spermatogenesis and also degenerative changes in primary and secondary spermatogonia, and integration of seminiferous tubules deranged were further observed (Fig. 5b, 5c, 5d).

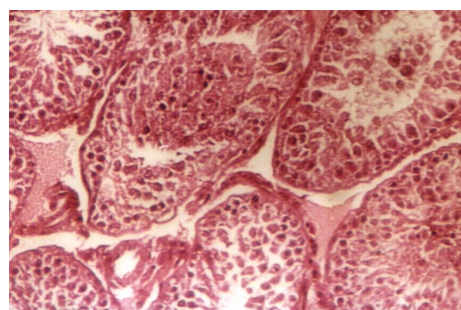
However mild regeneration of seminiferous tubules was observed when testis of mice treated with AC and HEAPE mixture (Fig. 5e, 5f, 5g).



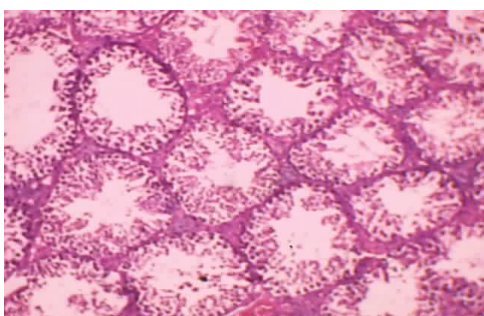
**Fig. 5a:** Control testis.



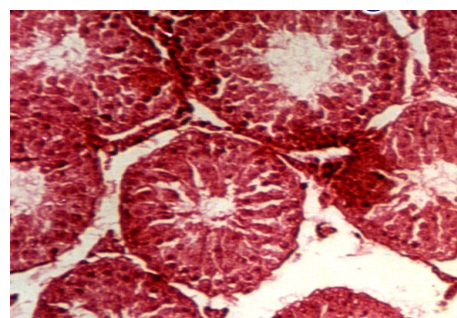
**Fig. 5b:** 1mg AC /48hr



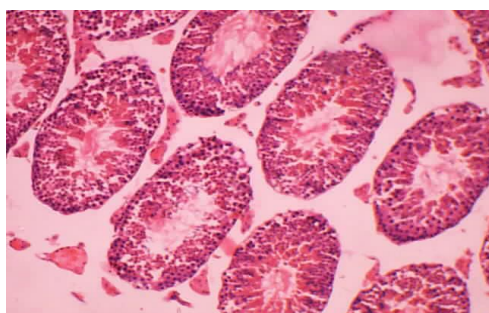
**Fig. 5e:** 1mg AC and HE /48hr .



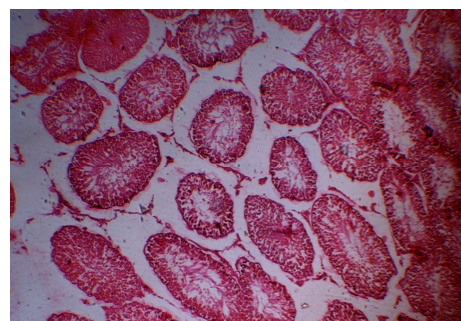
**Fig. 5c:** 2mg AC /48hr.



**Fig. 5f:** 2mg AC and HE /48hr.



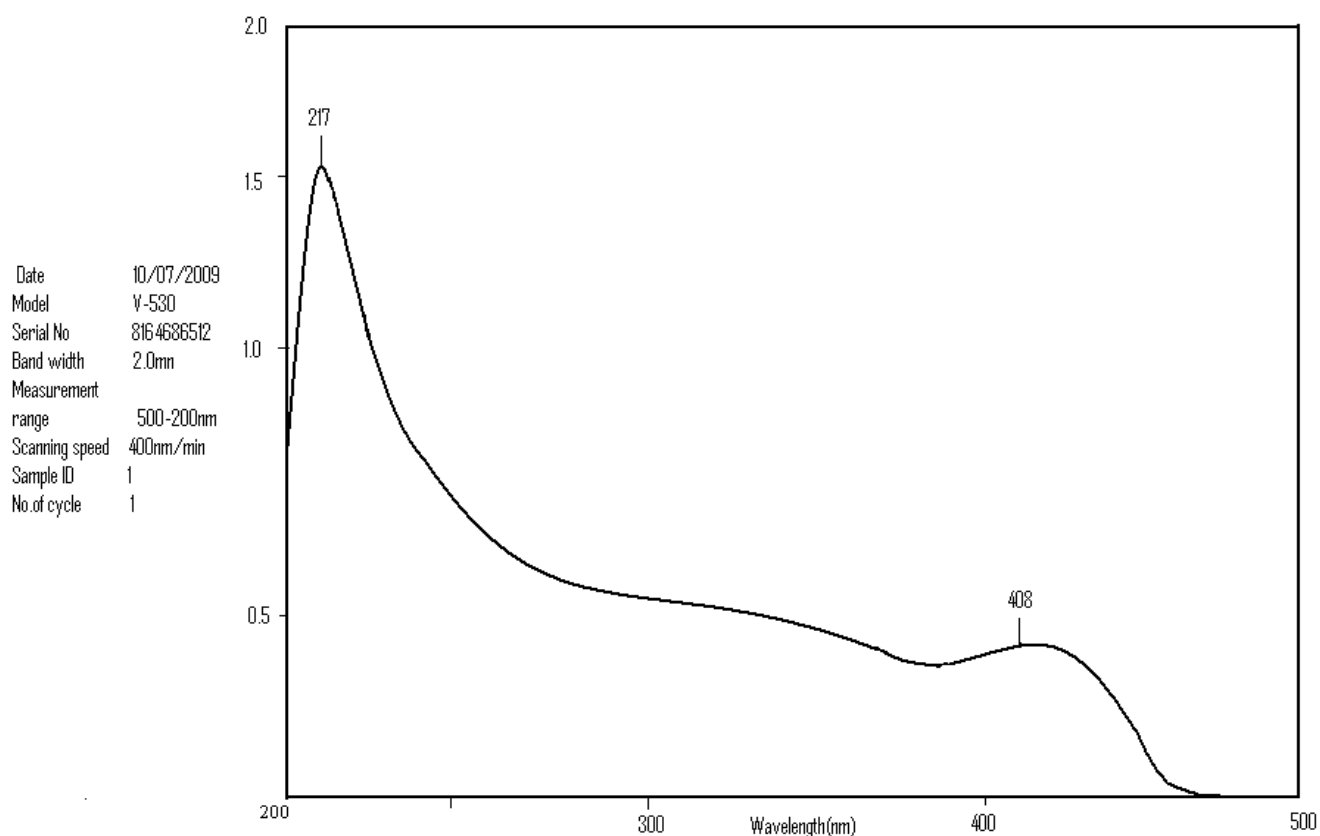
**Fig. 5d:** 3mg AC /48hr.



**Fig. 5g:** 3mg AC and HE /48hr.

*Note: Severe damage to Seminiferous tubules  
Left panel acrylamide sections and right panel AC+HE treated testis sections after 48 hrs.*

*Mild degeneration of Seminiferous tubules*



## DISCUSSION

Acrylamide has been evaluated for reproductive toxicity in multigenerational studies in rats. In the toxic studies with rat the increase of tumours was most evident in specific organs, e.g. adrenal gland, scrotal mesothelium. Acrylamide (10-20mg/kg body weight) caused testicular degeneration and spermatocytes chromosome aberrations in mice as observed by the formation of micronuclei in spermatids of rats (Laxminarasiah *et al.*, 2011). The tissue degeneracy of testis and hypertrophy with binucleated hepatocytes in liver was compared with the enzyme activities of GST and GPx protein levels and were further confirmed by immunological studies using their subunit specific and affinity protein antisera. These results of mice testis are almost identical to the effects of acrylamide on liver of animals.

In mice testis the acrylamide has induced only GSTs and not GPxs for the protection of cells from these insults (Table 1). The immunoblot analysis of mice testis cytosols suggested the elevated expression of GSTs due to the effect of acrylamide on exposure from 24 to 72hours. The GST and GPx produced on AC treatment may protect testicular tissue from the neurotoxicant, acrylamide. However the supplement of HE along with acrylamide showed reduced activities of GST and GPx indicated the production of reactive radicals scavengers in the systems and the active biomolecules that are present in the isolated HE may serve as best nucleophiles in mice testicular systems. In the presence of HE the reduced activities of GST and GPx may indicate the

suppression of all GST proteins including GPx produced  $\alpha$ -class GSTs.

At lethal doses of acrylamide (4 mg) the concentration GST was elevated. The Quantification of subunits in both control and treated tissue of testis on immunochemical analysis, and also by enzymatic assays in a dose-dependent manner, revealed that Yc and Yb of the  $\alpha$  and  $\mu$  class were expressed predominantly. The present study suggests that the induction of the above mentioned subunits on AC treatment plays a role in the multi drug resistance mechanism, and that these subunits serves as a markers of neoplasia. Acrylamide treated mice testis of 48 intervals showed more intensified changes which include, increase in the lumen of the seminiferous tubules, clear degenerative changes in seminiferous tubules, necrotic spermatids and atrophied seminiferous tubules, extensive interstitial oedema and pyknotic nuclei and arrest of spermatogenesis and also degenerative changes in primary and secondary spermatogonia. Other symptoms included are testicular atrophy and degeneration of germinal epithelium, arrest of spermatogenesis and also shows degenerative changes integration of seminiferous tubules. However the treatment of the acrylamide along with HE showed less effects on the degeneration of lumens, spermatids and spermatogonia. To avoid damage to testis the HE had an active principle of an aromatic alkenyl carboxylate. This molecule may serve as an antioxidant to rectify the mistakes of protein synthesis and modification of various organelles of testis.

The HE on HPLC analysis showed two peaks of retention at ~2.15min on reverse phase chromatography column and this peak had absorption maxima at 217 and 408nm. These spectra indicate that the HE contained a conjugated di-ene with a solet band porphyrin ring molecule which may serve as scavenger of oxygen radicals generated by acrylamide and prevent them from further propagation. Henceforth the present research proposes that the HE can serve as inhibitor for acrylamide induced damage and protect the testis from various effects.

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