Abstract
Deltamethrin, the synthetic pyrethroid and its metabolites is reported to have serious impacts on human health including neuro, nephro and hepatotoxicity. The present study discusses the effect of Deltamethrin in human hepatocytes and the ameliorative effect of selenium on reversing pesticide toxicity. HepG2 cells exposed with Deltamethrin confirmed toxicity in a dose dependent manner with IC values of approximately 265 mM. Membrane damage and loss of cytosolic integrity was evident from LDH leakage and NRU assay. Selenium was selected as the ameliorating agent owing to its association with hepatic enzymes which have considerable role in xenobiotic metabolism and anti oxidant status of the cell. Sodium selenite was toxic only at higher concentration with little or no morphological changes when determined by MTT assay. The role of Reactive oxygen species (ROS) was confirmed by DCFDA staining. Addition of selenium considerably increased the glutathione reserves which reduced oxidative damage produced by DMT which can be accounted as the major mechanism of hepato protection offered by Sodium selenite.

Keywords: Hepatotoxicity, Deltamethrin, MTT, DCFDA, HepG2.

INTRODUCTION
The use of pesticide is one of the most conferred topics in the agro fields of our economy. Ever since the green revolution the emphasis on agro products has increased drastically leading to both overuse and misuse of pesticides in an uncontrollable manner. The use of pesticides extends from cropping to harvesting and up to post harvest storage posing threats to farmers and others in a magnified manner. Since fertilizers and pesticides are closely related to economic stability and food safety an immediate retrieval of harmful pesticides is quite impossible. Another approach of ameliorating pesticide toxicity by food supplements or nutraceuticals can be appreciated.

Deltamethrin (DMT) is a type II synthetic pyrethroid, is widely used for pest management and to prevent rodents and pest in farms. The many deleterious effects of DMT such as neurochemical changes in mammalian brain [1,2] and interaction with γ-aminobutyric acid (GABA) receptor ionophore complex, leading to disturbed synaptic transmission and neuroexcitation resulting in behavioral anomalies [3,4,5]. The liver as known has proved to accumulate a greater concentration of metabolites since it is the major site of pyrethroid metabolism [6]. DMT is metabolized in the liver through hydrolytic ester cleavage by cytochrome P450's and the oxidative route [7].

Hepato toxicity is closely associated with depletion of intracellular antioxidants and glutathione content of the cell gains prior research significance in this aspect. GSH is an organic chemical that is found in plants and animals [8], being water soluble it is found mainly in cell cytosol and other aqueous phases of the living system [9, 10]. A decrease in reduced glutathione levels can shift the redox balance of the cell prompting it to undergo apoptosis or necrosis.

Selenium is considered and apt antioxidant in this case as it behaves both as an antioxidant and anti-inflammatory agent. This is because Se in its antioxidant role, notably as GPx, can (I) reduce hydrogen peroxide, lipid and phospholipids hydroperoxides, thereby dampening the propagation of free radicals and reactive oxygen species; (ii) reduce hydroperoxide intermediates in the cyclooxygenase and lipoxygenase pathways leading to inflammatory prostaglandins and leukotrienes and (iii)
modulate the respiratory burst, by removal of hydrogen peroxide and superoxide [11]. Selenium is a structural component of several enzymes with physiological antioxidant properties, including glutathione peroxidase (GSH-Px) and thioredoxine [12]. In recent years, there had been a great deal of studies carried out on selenium metabolism. In most of these studies the external selenium was given to experimental animals as sodium selenite [13]. The present study was objected to check the hepatoprotective effect of selenium against hepatotoxicity induced by Deltamethrin.

**Chemicals and Reagents**

Doulebecco’s Modified Eagle’s Media (DMEM), 3-(4,5-Dimethylthiazole-2yl)-2,5-Diphenyltetrazolium Bromide (MTT), Neutral Red (NR), Monochloro Bimane, 2’7’-dichloroflorescein Diacetate were purchased from Sigma Aldrich, USA. All other chemicals were of superior analytical grade from Invitrogen, Merck etc.

**Cell culture and trypsinization**

Hep G2 cell lines were purchased from National Centre for Cell Science (NCCS) Pune. The cells were maintained in DMEM containing 10% Fetal Bovine Serum (in-vitro) and sodium pyruvate (0.005g). At 70% confluence the cells were sub cultured for further studies.

**Pesticide preparation**

The pesticide selected was Deltamethrin 2.8% EC which was purchased from the local market The Deltamethrin (DMT) was diluted in Dimethyl Sulfoxide (DMSO) in 1:1 ratio.

1. **Induction of pesticide toxicity to HEP G2 cell line**

1.1 **MTT viability assay** [14]

A 96 well plate with HEP G2 was sub cultured and incubated for 24 hours. The pesticide, DMT was added using a range i.e. 25mM, 50mM, 100mM, 200mM, 400mM and 800mM. The plates were then incubated for 24 hours. MTT was added and the plate was incubated for 3 hours followed by 100µl of DMSO. The plates were then again incubated for half an hour and read using spectrophotometrically at 540nm and the percentage viability was calculated.

1.2 **Neutral Red Uptake assay** [15]

A plate that was priorily cultured with Hep G2 cell line was treated with the same range of DMT. After 24 hours of treatment, 10µl of NR was added and incubated for 3 hours tailed by 200µl of fixation buffer. It was then centrifuged at 3000rpm for 3 minutes; the supernatant was discarded and to the pellet 200µl of extraction buffer was added. This was then read using ELISA reader at 540nm. The percentage viability was calculated.

Using the above results the IC50 value was calculated and found to be 265mM.

2. **Determination of effective concentration of Sodium Selenite (NaSe) on HEP G2 culture**

2.1 **MTT viability assay** [14]

A plate cultured with Hep G2 cells were treated with a range of NaSe a concentration series of 0.005mM,0.01mM,0.05mM,0.1mM,0.2mM,0.4mM was taken. The plated were incubated for 24 hours and MTT was added following the same protocol as above. The percentage viability was calculated.

3. **Protective effect of Sodium Selenite on pesticide toxicity**

3.1 **MTT viability assay** [14]

A cultured plate of Hep G2 cell line was treated with a range of NaSe alone that includes the IC50 value and also the combination of DMT with the range of NaSe concentration was also added. The DMT concentration was fixed as the IC50 value i.e. 0.125mM NaSe, 0.25mM NaSe, 0.5mM NaSe, 265mM DMT+0.125mM NaSe, 265mM DMT+0.25mM NaSe, 265mM and DMT+0.5mM NaSe. After 24 hours the treated cells incubated for 3 hours after MTT addition and the protocol was followed. The percentage viability was calculated.

4. **Effect of sodium selenite on ROS generation by DM on Hep G2 cell line.**

The cell culture plate priorily treated with the sample combination for 24 hours was removed from the CO2 incubator and their media was removed followed by brief PBS wash. This wells with cells treated with sample is now ready for the following staining

4.1 **Intracellular GSH determination by MCB**

50µl of MCB1 stain was added into the respective wells and allowed to be incubated for 15minutes. After the incubation time the stain was removed and washed twice with PBS. It was then viewed under fluorescent microscope using blue filter.

4.2 **Intracellular ROS generation determination by DCFDA**

The wells that were washed with PBS were taken and 50µl of DCFDA was added and incubated for 30 minutes. Later the stain was removed and PBS wash was given twice to remove any excess stain. The wells were then viewed using blur filter of fluorescent microscope.

**RESULTS**

1. **Induction of pesticide toxicity to HEP G2 cell line**

1.1 **MTT viability assay**

![FIG 1.1 Cytotoxicity assay by MTT assay in Hep G2 cells following the exposure of various concentration (25-800mM) of pesticide DMT. With the x axis being the various concentrations of DMT and y axis the percentage viability. Values are of SD of three independent experiments.](image-url)
1.2 Neutral Red Uptake assay

FIG 1.2 Cytotoxicity assay by NRU assay in Hep G2 cells following the exposure of various concentration (25-800 mM) of pesticide DMT. With the x axis being the various concentrations of DMT and y axis the percentage viability. Values are of SD of three independent experiments.

2. Determination of effective concentration of Sodium Selenite on Hep G2 culture

2.1 MTT viability assay

FIG 2.1 Cytotoxicity assay by MTT assay in Hep G2 cells following the exposure of various concentration (0.005-0.4 mM) of NaSe. With the x axis being the various concentrations of NaSe and y axis the percentage viability. Values are of SD of three independent experiments.

FIG 2.1.1 Schematic representation of the morphological changes induced by various concentration of NaSe on HepG2 cell line. (a) Control, (b) 0.005 mM, (c) 0.01 mM, (d) 0.05 mM, (e) 0.1 mM, (f) 0.2 mM.

3. Protective effect of Sodium Selenite on pesticide toxicity

3.1 MTT viability assay

FIG 3.1 Cytotoxicity assay by MTT assay in Hep G2 cells following the exposure of various concentration of NaSe (0.125 mM, 250 mM, 500 mM) as well as combination of NaSe (the same concentration) with DMT (265 mM). (a) The combination of NaSe with DMT, (b) NaSe. With the x axis being the various concentrations and y axis the percentage viability. Values are of SD of three independent experiments.
FIG 3.1.1 Schematic representation of morphological changes induced by pesticide and combination of pesticide with Deltamethrin a) Untreated control cells b) cells

4. Effect of sodium selenite on ROS generation by DM on Hep G2 cell line.
4.1 Intracellular GSH determination by MCBI

Fig 4:1: MCBI localization of reduced glutathione (a) control cells showing baseline glutathione reserves (b) Selenium treated HepG2 cells showing increased glutathione content (c) Cells treated with Deltamethrin showing depletion of reduced glutathione (d) Selenium and Deltamethrin showing increase in glutathione content

4.2 Intracellular ROS generation determination by DCFDA

FIG 4.2 DCFDA staining for determination of ROS generation. (a) Control, (b) DMT (c) Sodium selenite (d) NaSe+DMT: Increase in ROS production is Evident in DMT treated (b) which is reduced by addition of sodium selenite (d)

DISCUSSION
Xenobiotic metabolism and accumulation results in shift of oxidative balance of almost all mammalian cells of which hepatocytes are mostly affected. In the present study we determined the toxic effects of a Deltamethrin, pyrethroid class of insecticide. Even though Type II pyrethroids, including Deltamethrin, produce characteristic effects of choreoathetosis (sinuous writhing) and salivation, also known as CS Syndrome [16] least is reported regarding possible hepatotoxicity which formed the major objective of the present study

Limited information is available on disposition of pyrethroids [17, 18, 19, and 20]. Deltamethrin is rapidly absorbed when administered orally or intraperitoneally and enters the circulatory system and reaches liver [6]. We used HepG2 cells for assessing the possible hepatotoxicity produced by Deltamethrin and from the results it can be found that 265mM which falls in normal range of pesticide exposure. Comparable values were obtained for neutral red
assay. Increase in LDH leakage clearly depicts loss of membrane integrity as a result of pesticide exposure. The viability assessment confirms dose dependent toxicity of Deltamethrin and our objective was to ameliorate that by using selenium.

Selenium or sodium selenite was selected mainly to its involvement in hepatic glutathione metabolism and role in maintaining redox balance. Selenium forms the indispensable part of glutathione peroxidase which is required for oxidation of reduced glutathione to oxidized one [21, 22]. Since selenium is reported to be toxic at higher concentration, we performed a dose dependent study of different concentration of selenium which gave and IC 50 values of approximately 225µM. selenium is non toxic at lower concentration but the risk increases with accumulation as increased concentration leads to formation of superoxide ions with thiols [23]. It can be observed that addition of higher concentration of sodium selenite does not alter the viability to a higher extent. Hence this part concludes that the increase in selenium concentration doesn’t make a marked difference in cell viability.

The role of reactive oxygen species in hepatotoxicity induced by Deltamethrin was determined by fluorescent tagging method using DCFDA. The significance and sensitivity of DCFDA in measurement of ROS is well described by workers like Ameziane, 2010 [24]; LaBel et al, 1992; Wu and Arthur, 2013. Our results show nearly 80% increase in fluorescence intensity when cells were exposed with pesticides which confirms generation of intracellular ROS. Selenium alone at 0.125 mM concentration was found to produce only negligible amount of ROS. Addition of selenium to Deltamethrin treated cells decreased ROS generation. ROS generation accounts to cell damage and death when intracellular antioxidant levels are decreased to a greater extent. As per our hypothesis addition of selenium enhanced the utilization of reduced glutathione, the major antioxidant compound of the cells and hence most of the ROS are scavenged by glutathione which resulted in decreased fluorescence. Thiol specific dye monochlorobimane also confirms decrease in intracellular glutathione of HepG2 cells treated with Deltamethrin which may be due to conversion in oxidized form.

As per recent findings of Aljendra and coworkers the metabolite 4’ OH Deltamethrin is nearly 400 times toxic than Deltamethrin that increases significance of the present study. The higher IC 50 values of Deltamethrin can be justified such that the toxicity is increased many fold after phase I and II metabolism of pesticide [26] and liver being the site of metabolism the toxicity can increase up to several times.

CONCLUSION
In conclusion, this study is the first to report the hepatotoxic effect of Deltamethrin in human cells and the correlation between glutathione and oxidative injury produced by DMT. The use of selenium was ascertained to reduce the radical generated toxicity and can in turn find applications as supplement to compensate pesticide induced oxidative damage. The present studies advance the significance of antioxidants in protection against pesticide toxicity and can draw significant insights regarding in vivo and invitro data comparison.

ABBREVIATIONS
DMEM : Dulbecco’s Minimal Eagle’s Media
DMSO : Dimethyl Sulfoxide
DMT : Deltamethrin
EC : Emulsifiable Concentrate
FBS : Fetal Bovine Serum
GPx : Glutathione Peroxidase
GSH : Reduced Glutathione
Mg : Milligram
mM : Millimolar
MTT : 3-(4,5-Dimethylthiazole-2-yl)-2,5-
Diphenyltetrazolium Bromide
NaSe : Sodium Selenite
Nm : Nanometer
OD : Optical Density
PBS : Phosphate Buffered Saline
ROS : Reactive Oxygen Species

REFERENCE

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