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Protective effect of *Asteracantha longifolia* against carbon tetrachloride and paracetamol induced oxidative stress and lipid peroxidation in mice

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Abstract
The main objective of this study was to investigate the antioxidative effect of *Asteracantha longifolia* extract against carbon tetrachloride (CCl_4) and paracetamol induced oxidative damage in ICR mice. Hepatotoxicity was induced by a single intraperitoneal dose of CCl_4 (0.5 ml/kg) and paracetamol (300 mg/kg, orally) after a 16 h fast. An aqueous extract of *Asteracantha* was administered to mice on a pre and post-treatment basis. A significant increase in glutathione peroxidase and a significant reduction in glutathione reductase and glutathione-S-transferase concentrations were observed in CCl_4 and paracetamol control groups compared to normal control. A significant increase in malondialdehyde formation was observed in the liver cytosol and serum in both toxin control groups. Pre- and post-treatment with *Asteracantha* significantly improved the antioxidant enzyme profiles against CCl_4 and paracetamol control groups. Overall results indicate that aqueous extract of *Asteracantha longifolia* could ameliorate oxidative stress induced by CCl_4 and paracetamol in ICR mice.

Keywords: *Asteracantha longifolia*, carbon tetrachloride, paracetamol, oxidative stress, lipid peroxidation

Introduction
The liver is the primary organ responsible for the metabolism of substances such as carbohydrates and lipids, synthesis of many compounds such as cholesterol, blood clotting factors and detoxification and excretion of various endogenous and exogenous xenobiotics. Since many physiological activities are performed by the liver, it results in the generation of highly reactive free radicals that covalently binds to membrane lipids leading to lipid peroxidation. Lipid peroxidation causes alteration of the membrane permeability and tissue damage. However, liver contains an inbuilt antioxidant system that consists of superoxide dismutase (SOD), tissue glutathione (GSH) etc. and they protect the tissue from free radical attack. Due to the excessive production of reactive oxygen species, the antioxidant system is overburdened resulting in organ damage. In order to protect the liver from oxidative stress, it is important to strengthen the inbuilt protective mechanisms by the exogenous administration of antioxidants (Jo *et al*, 2016) [1].

Previous studies conducted in our laboratory identified the antioxidant potential of *Asteracantha longifolia* against carbon tetrachloride (CCl_4) and paracetamol induced hepatotoxicity (Hewawasam *et al*, 2003) [2]. We provided evidence that the aqueous plant extract of *Asteracantha longifolia* could protect the liver by improving the reduced glutathione status in the liver tissue. *Asteracantha longifolia* Linn that belongs to the family Acanthaceae is commonly known as "Neeramulliya". It is found in Sri Lanka and India. It is grown in the dry zone and also commonly found in the low country especially in ditches and in marshy lands. *Asteracantha* is commonly used as a decoction by traditional ayurvedic medical practitioners in Sri Lanka and it acts as a diuretic and an antidysenteric, as a treatment option for renal calculi and also hepatic derangements (Jayaweera, 1981) [3].

Carbon tetrachloride is a well-known hepatotoxin that is responsible for apoptosis, necrosis and hepatocellular damage. Chronic administration of CCl_4 leads to fibrosis, cirrhosis as well as hepatocellular carcinoma. Furthermore, CCl_4-induced liver injuries occur due to the activation of cytochrome P450 enzyme system that produce highly reactive intermediates mainly trichloromethyl free radical (CCl_3) and trichloromethyl peroxy radical ($CCl_3OO^{\bullet}$). These radicals can bind to cellular molecules such as proteins, lipids and nucleic acids promoting lipid peroxidation and impairing lipid metabolism. It may also initiate hepatic cancer. Therefore, the inhibition of synthesis as well as activation of free radicals is considered

as a very important factor for the prevention and treatment of CCl_4 mediated liver injury (Jo *et al*, 2016) [1].

Paracetamol is an over the counter analgesic and antipyretic that is safe and effective at therapeutic doses. However, when ingested at an excessively high dose, paracetamol can cause severe liver injury and acute liver failure. Development of liver injury by the administration of paracetamol is attributed to the formation of a highly reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), through oxidative metabolism mediated mainly by the hepatic enzyme, cytochrome P450. Generally, NAPQI is detoxified by the action of reduced glutathione (GSH). However, after the administration of a hepatotoxic dose of paracetamol, liver GSH levels are depleted hence NAPQI covalently binds primarily to cystein groups of proteins as 3-(cysteine-S-y) paracetamol adducts. Already published data suggest that mitochondrial dysfunction in the hepatocyte may be an important mechanism involved in the paracetamol induced hepatotoxicity (Baali *et al*, 2016) [4].

The present study was conducted to determine the antioxidative effect of *Asteracantha longifolia* against carbon tetrachloride and paracetamol induced hepatotoxicity. Here, we report that *Asteracantha longifolia* exhibited remarkable hepatoprotection against carbon tetrachloride and paracetamol induced hepatocellular injury, potentially by the amelioration of lipid peroxidation induced by oxidative stress and by improving the antioxidant enzyme levels in the hepatocyte.

Methodology

Experimental animals

Healthy male ICR mice, 6-8 weeks old and weighing 30-35 g, were allowed free access to water and pelleted food *ad libitum*. All animals were fasted for 16 h before administration of the hepatotoxin. All protocols used in this study were approved by the ethics committee of the University of Ruhuna, Sri Lanka, guided by the CIOMS international guiding principles of biomedical research involving animals.

Chemicals

Paracetamol was a gift from the Sri Lanka Pharmaceutical Manufacturing Corporation. 5, 5'- dithiobis (2-nitrobenzoic acid), thiobarbituric acid, glutathione reductase, cumine hydroperoxide, NADPH and 1-chloro 2, 4 dinitrobenzene were purchased from Sigma (St. Louis, Missouri). N-Acetylcysteine (NAC) was obtained from the Teaching Hospital, Karapitiya, Galle, Sri Lanka. All other reagents were commercially available and of reagent grade.

Preparation of the plant extract

Whole plants of *Asteracantha longifolia* were collected from the Galle district in the Southern province of Sri Lanka. The sample was authenticated by comparison with the herbarium specimen preserved at the National Herbarium in the Botanical Gardens, Peradeniya, Sri Lanka. A voucher specimen was deposited at the Department of Biochemistry, University of Ruhuna, Sri Lanka.

Whole plants of *Asteracantha longifolia* were cut into small pieces and dried at 40 °C for two days. The normal therapeutic dose of humans extrapolated to mouse was used (Dhawan and Srimal, 1998) [5]. 2.625 g of the dried plant material was refluxed in 30 mL of distilled water for 1 h and concentrated to 20 mL. Each mouse was administered a dose of 0.9 g kg^{-1} orally by gavage. The extract was prepared daily from the dried plant material.

Treatment of animals

Control groups

Mice were divided into two groups of 10 animals in each. The first group served as the normal control group and received distilled water orally by gavage. The second and third groups were treated with the *Asparagus* and *Vetiveria* extracts alone for 7 days. Animals were killed 7 days after the administration of the plant extract.

Carbon tetrachloride-induced hepatotoxicity

Mice were randomly divided into six groups (groups 3-8) of 10 animals in each. A single intraperitoneal dose of CCl_4 was injected (0.5 mL/kg in olive oil, CCl_4: olive oil 1:10) in each animal after a 16 h fast. In groups 3 and 4 the animals were killed 24 h and 4 days, respectively, after the administration of CCl_4. Animals in group 5 were administered *Asteracantha longifolia* extract half an hour after the administration of a single dose of CCl_4 and were killed 24 h later. The same procedure was carried out for group 6 but instead of killing after 24 h, they were given the extract alone for a further two days at 24 h intervals (post-treatment). They were killed on the fourth day. Groups 7 and 8 were administered the *Asteracantha longifolia* extract daily for seven days and on the seventh day a single dose of CCl_4 was injected half an hour after the administration of the plant extract. The mice were killed after 24 h and 4 days, respectively.

Paracetamol induced hepatotoxicity

Mice were randomly divided into four groups (groups 9-12) of 20 animals each. 300 mg/kg of paracetamol (dissolved in saline and heated at 60 ˚C) was administered orally after a 16 h fast. Group 9 was given paracetamol alone and killed 4h later. Group 10 received the same dose of paracetamol and half an hour later 500 mg/kg of NAC was given orally. The mice were killed 4 h later. In the group 11, *Asteracantha longifolia* extract was administered instead of NAC. *Asteracantha longifolia* was administered for 7 days in group 12 and on the seventh day paracetamol was administered half an hour after the administration of the plant extract. Animals were killed 4h later.

Estimation of lipid peroxidation

The extent of lipid peroxidation was estimated in liver homogenates/serum by the measurement of malondialdehyde (MDA) formation using thiobarbituric acid method. Method of Muriel *et al* (2001) [6] as described in Okawa *et al* (1979) [7] was used. Malondialdehyde (MDA) is reacted with thiobarbituric acid at 95 °C and the absorbance of the pink coloured product was measured at 532 nm against a reagent blank.

Estimation of antioxidant enzymes in the liver

Glutathione reductase (GR, EC 1.6.4.2) and glutathione peroxidase (GPx, EC 1.11.1.9) levels were assayed in the cytosolic fraction. GR assay is based on the oxidation of NADPH to $NADP^+$ catalysed by a limiting concentration of glutathione reductase. GPx catalyses the reduction of hydrogen peroxide to water and organic peroxides (ROOH) to the corresponding stable alcohols, (R-OH) using glutathione as a source of reducing equivalents. Oxidized glutathione produced upon reduction of organic peroxide by cellular GPx

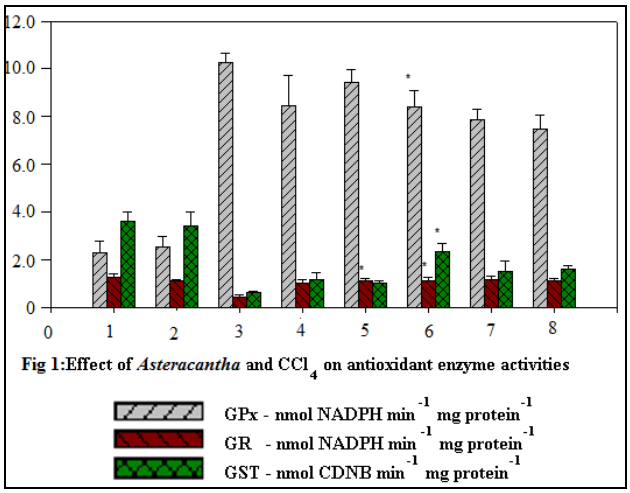
is recycled to its reduced state by glutathione reductase. The enzyme activity was determined by measuring the disappearance of NADPH at 340 nm and was expressed as nmoL of NADPH oxidized per minute per mg protein. Glutathione-S-transferase (GST, EC 2.5.1.18) activity was measured by the method as described by Athar *et al* (1989) [8] and Ansar *et al* (1999) [9]. Assay is based on the conjugation of 1-chloro, 2, 4, dinitrobenzene (CDNB) with reduced glutathione producing a dinitrophenyl thioether and chloride ion. Product formation is accompanied by the appearance of an absorption band at 340 nm.

Statistical analysis

The results were evaluated by one-way analysis of variance and Tukey's multiple comparison test. A probability (*P*) value of less than 0.05 was considered significant.

Results

Figures 1 and 2 summarize the effect of the plant extract *Asteracantha longifolia* on the antioxidant enzyme activity and lipid peroxidation against CCl_4 induced hepatocellular injury. Only GPx activity was increased to 344.8 and 265.8 percent 24 h and four days after the administration of CCl_4. GR and GST activities were reduced to 92.2, 21.1 and 83.2, 67.8 percent respectively, 24 h and four days after the administration of CCl_4.



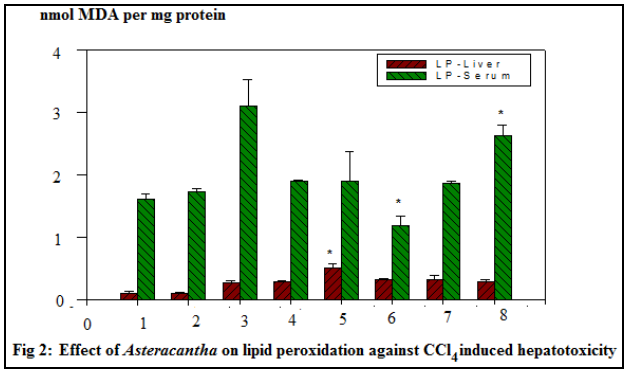
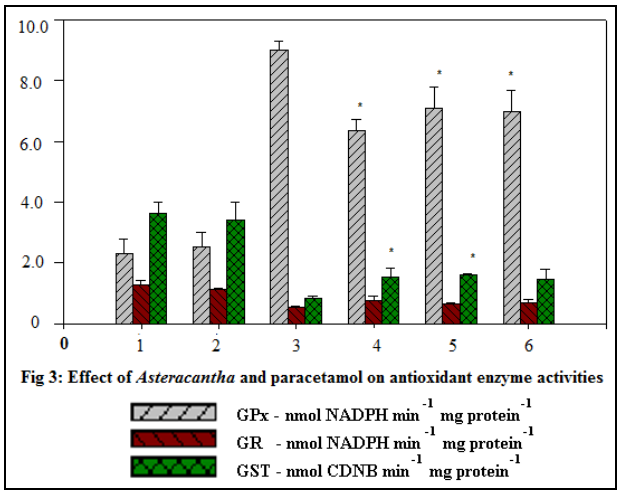


Fig 1 & 2: n=10 mice in each group. Group 1: Normal control group, treated with distilled water; Group 2: Plant extract (0.9 g/kg, p.o) for 7 days. Group 3: a single dose of carbon tetrachloride (0.5 ml/kg in olive oil, ip) and sacrificed 24 h later; Group 4: a single dose of carbon tetrachloride (0.5 ml/kg in olive oil, ip) and sacrificed 4 days later; Group 5: Post-treatment, sacrificed 24 h later; Group 6: Pre-treatment, sacrificed 24 h later; Group 7: Post-treatment, sacrificed 4 days later; Group 8: Pre-treatment, sacrificed 4 days later. Results given as mean ± S.E.M.



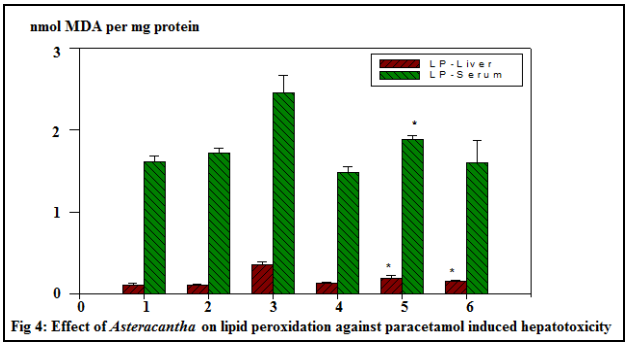


Fig 3 & 4: n=20 mice in each group. Group 1: Normal control group, treated with distilled water; Group 2: *Asteracantha* control (0.9 g/kg, p.o) for 7 days. Group 3: a single dose of paracetamol (300 mg/kg in saline, orally) and sacrificed 4 h later; Group 4: a single dose of paracetamol + N-acetyl cysteine (500 mg/kg) and sacrificed 4 h later; Group 5: Post-treatment with *Asteracantha*, sacrificed 4 h later; Group 6: Pre-treatment with *Asteracantha*, sacrificed 4 h later; Results given as mean ± S.E.M.

In the *Asteracantha* control group, there were no significant changes in the antioxidant enzyme levels compared to the normal control. Although all enzyme parameters were improved in *Asteracantha* treated mice, a significant change in all three enzyme levels was observed only in *Asteracantha* pre-treated group 24 h after the administration of CCl_4.

On comparing the malondialdehyde formation in the CCl_4 control group with the normal control group, a percentage increase in 153.3 and 163.6 were observed 24 h and four days later in the liver cytosolic fraction. However, a percentage increase in 92.1 and 18.2 was observed in the serum malondialdehyde levels 24 h and four days after the administration of CCl_4. A significant improvement in the malondialdehyde formation was observed in the serum in *Asteracantha* pre-treated mice 24 h and 4 days after the administration of CCl_4.

Figures 3 and 4 summarizes the effect of plant extracts *Asteracantha* on antioxidant enzyme levels and lipid peroxidation against paracetamol induced hepatocellular injury. When considered the GPx level in plant extract treated

groups, a significant reduction in the enzyme levels were observed in *Asteracantha* pre and post-treated groups four hours after the administration of paracetamol. N-acetyl cysteine control group showed a significant improvement only in the GPx and GST activities but not in the GR level. A significant improvement in the GST concentration was also observed in the *Astercantha* post treated group compared to the paracetamol control group.

Compared to the normal control, a significant increase in the malondialdehyde formation was observed both in the liver and serum in mice treated with paracetamol alone. When post treated with *Asteracantha*, both levels were reduced significantly. A significant reduction was observed only in the liver cytosolic fraction in the pre-treated mice four hours after the administration of paracetamol.

Discussion & conclusion

There are two main defense mechanisms that act against xenobiotic induced toxicity in the body; drug metabolizing enzymes and antioxidant systems. During the metabolism of xenobiotics, electrophiles, radicals and reactive oxygen species (ROS) are produced as intermediates or by-products. These molecules induce lipid peroxidation and oxidation of DNA and other cellular components that result in the production of acute and chronic tissue injuries, carcinogenesis and aging. Antioxidants such as GSH, vitamins and antioxidant enzymes play a major role in the prevention of the accumulation of these harmful oxidants in the cell (Enomoto, 2001) [10].

According to Zorov *et al* (2014) [11], oxidative damage induced by the loss of homeostasis functioning and the loss of mitochondria can cause irreversible cell damage. However, by maintaining an adequate concentration of intracellular antioxidants, and by the action of repair systems the antioxidant enzymes are stimulated to reverse the initiation of oxidative damage. Antioxidant enzymes produced in excess scavenge excess free radicals produced thereby contributing to a decrease in oxidative damage. On the other hand a decrease in the levels of antioxidants lead to an increase in oxidative damage.

The antioxidative defense enzymes have been identified as playing an important role in the maintainance of physiological levels of oxygen and hydrogen peroxide and also for eliminating peroxides produced by the exposure to xenobiotics or drugs. However, additional protective mechanisms of antioxidants may be of great importance when the endogenous protection may not be complete or when the formation of reactive oxygen species is excessive. Therefore, medicinal plants possessing antioxidative properties have been used to prevent hepatocellular damage induced by oxidative stress.

In this study, antioxidative effect of *Asteracantha longifolia* was evaluated by the determination of glutathione peroxidase, glutathione reductase and glutathione S- transferase enzyme levels in the liver homogenate of ICR mice and by the malondialdehyde formation in the liver cytosol and serum.

Glutathione peroxidase is widely distributed in almost all tissues within cells, but the highest concentrations are found in hepatocytes. The predominant subcellular distribution is in the cytosol and mitochondrion. Therefore, glutathione peroxidase is the main scavenger of hydrogen peroxide in these subcellular organelles. The activity of the enzyme is mainly dependent on the continuous supply of reduced glutathione (Young, 2001) [12]. Therefore, in the presence of an oxidative stress, an elevation of GPx activity is commonly observed. Toxic metabolites produced by the metabolism of xenobiotics are efficiently detoxified by the high levels of GPx. Yagi *et al* (Yagi, 1996) [13] reported that, the marked protection by GPx against oxidative damage in cells is by over expression through transfection. It was also explained that over expression of extra cellular GPx can protect against damage by increased scavenging of the released reactive oxygen species as well as by inhibition of activation of inflammatory leukocytes, which play an important role in paracetamol-induced hepatotoxicity (Goldin *et al*, 1996) [14].

Glutathione reductase, a secondary antioxidant enzyme is used for the regeneration of reduced glutathione from oxidized glutathione. It has a similar tissue distribution to glutathione peroxidase. The higher ratio of reduced to oxidized glutathione is usually maintained as a result of the activity of the enzyme glutathione reductase (Young, 2001) [12]. Many studies have shown that GR is susceptible to oxidative damage from excessive free radical generation *in vitro* and *in vivo* (Barker *et al*, 1996) [15]. It may be the reason for the very low reduced glutathione level observed in paracetamol control group.

Drug metabolizing enzymes, such as glutathione-S-transferase, work in concert with antioxidant systems by metabolizing electrophiles and xenobiotics, and some of the molecules involved in the two defense mechanisms are induced simultaneously in response to xenobiotic exposure (Enomoto, 2001) [10]. The cytosolic glutathione-S-transferase catalyses the conjugation reaction of GSH and electrophilic substances, and therefore, has an important role in the detoxification of electrophilic toxicants (Kaplowitz *et al*, 1980) [16].

A percentage increase in 344.8 and 265.8 was observed in glutathione peroxidase (GPx) activity in CCl_4 intoxicated mice 24 h and 4 days after the administration of CCl_4, while a percentage reduction in 92.2, 21.1 and 83.2, 67.8 respectively was observed in glutathione reductase (GR) and glutathione-S-transferase (GST) activities. *Asteracantha longifolia* significantly improved the changes mediated by the hepatotoxins on the levels of glutathione peroxidase, reductase and S-transferase. The observed rise in the glutathione peroxidase activity could be an adaptive response to high levels of free radicals formed in response to toxic insult. When the activity of GPx was considered, pre-treatment showed better results than the post-treatment. But the activity of GR was opposite to that of GPx. Since administration of plant extracts significantly increased the GR activity, it can be assumed that the active components in plants may be responsible for the reduction in the formation of peroxynitrite or hydroxyl radicals thereby minimizing the oxidative stress. Although a definite pattern is not observed in the GST concentration, all values were improved compared to respective control groups. Results shown in this study on antioxidant enzyme concentrations are compatible with previously published data in Hewawasam *et al* (2003) [2] where reduced glutathione concentrations were improved significantly in *Asteracantha* treated groups in both CCl_4 and paracetamol intoxicated mice. A similar pattern was explained by Jodynis-Liebert *et al* (2000) [17] where a reduction in the GPx and GR activities were observed in plant treated groups. GST activities were increased significantly in tannic acid treated mice as explained in Athar *et al* (1989) [8].

Guttridge and Halliwell (1990) [18] explained that, cell or tissue destruction by radicals or otherwise can lead to more

lipid peroxidation because antioxidants in cells get diluted and transition metal ions that can stimulate the peroxidation process are released from disrupted cells. This stimulation of lipid peroxidation can make a significant contribution to injury. In the process of lipid peroxidation, malondialdehyde (MDA) is formed as a catabolic product. The adverse effect on hepatic GSH status was associated with a substantial increase in hepatic MDA level, an indirect index of lipid peroxidation.

All three extracts could reduce the level of malondialdehyde both in the liver cytosolic fraction and in serum. A percentage increase in malondialdehyde concentration by 153.3 and 163.6 were observed in liver and serum 24 h and 4 days after the administration of CCl_4. Many components cited in the literature as being beneficial against carbon-tetrachloride mediated lipid peroxidation, act either via a decreased production of free radicals from carbon tetrachloride or through the antioxidant activity of the protective agents themselves (Ganie, 2011) [19]. 227.1 and 52.1 percent increase in MDA concentration was observed in paracetamol control group compared to normal control. *Asteracantha longifolia* reduced the malondialdehyde formation in both CCl_4 and paracetamol intoxicated mice. Muriel *et al* (2001) [6] also explained an increase in liver MDA activity by 2.5 fold the normal control in the CCl_4 control group and a significant reduction in drug treated groups.

Both CCl_4 and paracetamol must be microsomally metabolized before their toxic properties are manifested. In addition, the cascade of cellular injury involving free radicals is a complicated process involving multiple activities of enzymatic and non-enzymatic antioxidants. Therefore, *Asteracantha* may exert its action against CCl_4 and paracetamol induced liver damage by impairing the metabolism of toxins through inhibition of the activities of microsomal enzymes or by modulating the activity of a variety of enzymatic and non-enzymatic antioxidants. In conclusion, results presented here show clearly that aqueous extract of whole plant of *Asteracantha longifolia* could enhance the antioxidant enzyme profile in the liver and exert protection against carbon tetrachloride and paracetamol induced liver injury.

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