Epaltes divaricata in paracetamol poisoning: A biochemical and histopathological investigation

Hewawasam RP[1], Jayatilaka KAPW [1], Mudduwa LKB[2]

Department of Biochemistry[1], Department of Pathology[2], Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka.

Correspondence: Dr. R.P. Hewawasam
Department of Biochemistry, Faculty of Medicine, Karapitiya, Galle, Sri Lanka.
e-mail: ruwaniph@yahoo.com

Abstract

***Introduction and Objective**:* In the practice of traditional ayurvedic medicine in Sri Lanka, a number of herbs have been recognized for their potential benefits in the treatment of liver disorders. This study was conducted to investigate the protective effect of *Epaltes divaricata* plant extract in paracetamol induced hepatotoxicity in mice.

Methods: ICR mice (n=20) were treated with acetaminophen at a single dose of 300 mg/kg (after a 16h fast) to induce hepatotoxicity. Drug control group and pre and post-treated groups were administered 0.9 g/kg of *Asparagus falcatus* orally. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatise (ALP) and liver reduced glutathione (GSH) levels were determined. Liver damage was also assessed histopathologically. The effect of the plant extract was compared with *N*-acetyl cysteine.

***Results**:* Acetaminophen produced liver damage, as manifested by a significant rise (P<0.001, one-way ANOVA) in serum ALT, AST, and ALP, and a reduction (P<0.001) in GSH as compared to respective controls. All enzyme activities and liver GSH were significantly improved in *Epaltes* treated mice, with post-treatment providing better results than pre-treatment. Histopathological changes were compatible with the observed biochemical abnormalities.

***Discussion**:* This study shows the ability of the aqueous extract of *Epaltes divaricata* to preserve liver functions in mice treated with high dose of paracetamol.

Introduction

Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Drug discovery from medicinal plants leads to the isolation of early drugs such as cocaine, codeine, digoxin, and quinine, in addition to morphine, of which some are still in use. It continues to provide new and important leads against various pharmacological targets including cancer, HIV/ AIDS, Alzheimer's disease and malaria. Several natural product drugs of plant origin have either recently been introduced to the United States market, including arteether, galantamine, nitisinone, and tiotropium, or are currently involved in late-phase clinical trials (1). There are a number of natural remedies that have claimed to possess curative effects in liver disorders. Recent progress in the study of traditional drugs has resulted in the isolation of numerous active compounds, including antihepatotoxic constituents. Our previous studies provided scientific evidence for the use medicinal plants as hepatoprotective agents against both carbon tetrachloride and acetaminophen-induced hepatotoxicity in mice (2-4).

Figure 1: *Epaltes divaricata* (Sinhala: Heen Mudamahana) Family: Compositae

Acetaminophen (paracetamol) is one of the most widely used non-narcotic analgesic and antipyretic agents available over the counter (5). Although considered safe at therapeutic doses, in overdose, acetaminophen produces centrilobular hepatic necrosis which can be fatal. Acetaminophen is frequently misused and its indiscriminate ingestion can lead to poisoning and life-threatening hepatotoxicity (6). Every year in the United States, overdoses of acetaminophen causes acute liver failure in as many as 800 people, one third of whom die (7). The incidence of self-induced poisoning with acetaminophen is not a significant problem in the developing world as compared to the developed countries (8). However, the number of admissions due to paracetamol poisoning has increased significantly over the last decade. According to a study conducted at the National Poisons Information Centre, of the 992 reported cases of poisoning to the National Hospital Sri Lanka due to medicines, 845 cases were due to ingestion of paracetamol (85%) either alone or with another drug or poison from 2003-2005 (abstract in www.asiatox.org). N-Acetyl cysteine (NAC) is the standard therapy for the treatment of acetaminophen overdose in patients. The primary role of NAC in the treatment of acetaminophen toxicity is in the replacement of intracellular stores of hepatic GSH which allows for detoxification of the electrophile N-acetyl-p-benzoquinone imine (NAPQI), the reactive metabolite formed during metabolism of acetaminophen (9). NAC was used as the reference drug in the present study.

Epaltes divaricata (Family- Compositae, Figure 1), a divaricately branched annual herb, is found in Sri Lanka, India, Myanmar, Java and China. It is commonly known as "Heen Mudamahana" in Sinhala. *Epaltes* is used in traditional ayurvedic medicine to alleviate jaundice, urethral discharges and acute dyspepsia. It is also regarded as a diaphoretic and a diuretic. *Epaltes divaricata* is widely used in Sri Lanka not only as an ayurvedic medicine but also as a delicacy in villages. The objective of the present study was to evaluate the hepatoprotective and antioxidative effects of *Epaltes divaricata* against acetaminophen (paracetamol) induced hepatotoxicity in mice.

Methods

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 Review Committee of the Faulty of Medicine, University of Ruhuna, Sri Lanka, guided by the CIOMS international guiding principles of biomedical research involving animals.

Chemicals

Diagnostic kits for serum alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1) and alkaline phosphatase (ALP, EC 3.1.3.1) were purchased from Randox (UK). Acetaminophen was a gift from the Sri Lanka Pharmaceutical Manufacturing Corporation. 5,5'-Dithiobis (2-nitrobenzoic acid) was purchased from Sigma (St Louis, MO). N-acetyl cysteine (NAC) was obtained from the Teaching Hospital, Karapitiya, Galle, Sri Lanka. All other chemicals were commercially available and of reagent grade.

Preparation of the plant extract

Epaltes divaricata plants were collected from the Galle district in the Southern Province. 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.

Epaltes plants were cut into small pieces and dried at 40^{0}C for two days. The normal therapeutic dose of humans extrapolated to mouse was used (10). The dried plant material weighing 2.625 g was refluxed in 30 mL of distilled water for 1h and concentrated to 20 mL. Each mouse was administered a dose of 0.9 g/kg 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 20 animals in each. The first group (Group 1) served as the normal control group and received distilled water orally. The second group (Group 2) was treated with the *Epaltes* extract for 7 days. Animals were killed 7 days after the administration of the plant extract.

Paracetamol induced hepatotoxicity

Mice were randomly divided into four groups (Groups 3-6) of 20 animals in each. Paracetamol 300 mg/kg (dissolved in saline and heated at 60 ^{0}C) was administered orally after a 16 h fast. Group 3 was given paracetamol alone and was killed 4h later. Group 4 received the same dose of paracetamol and half an hour later NAC was given orally at 500 mg/kg. The mice were killed 4 h later. In group 5, *Epaltes* extract was administered instead of NAC (Post-treatment). *Epaltes* extract was administered for seven days in group 6 and on the seventh day paracetamol was administered orally half an hour after the administration of the plant extract (Pre-treatment). Animals were killed 4h after the administration of paracetamol in each group.

Assessment of liver damage

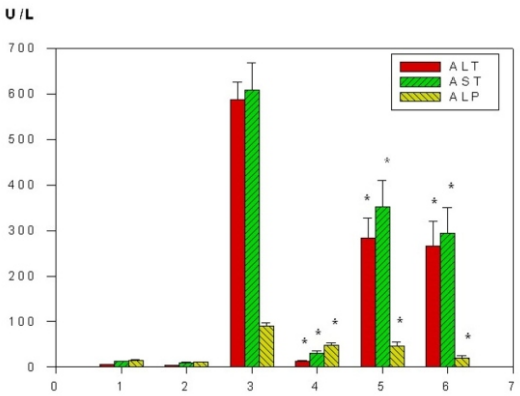
Blood was drawn by cardiac puncture under ether anaesthesia to determine ALT, AST and ALP activity. Liver tissues were excised, weighed and a section of the liver was fixed in 10% buffered formalin for histopathological assessment of liver damage. A liver section was homogenized and used for the determination of reduced glutathione (GSH) level in the liver. Serum ALT, AST and ALP activities were measured using an assay kit from Randox, UK (11). The liver GSH level was estimated by the method of Jollow *et al* (12). Histological sections of the formalin fixed liver tissue were stained with haematoxylin and eosin.

Statistical analysis

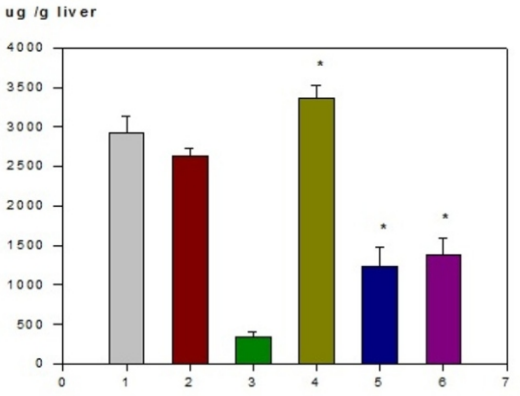
The results were analysed by one-way ANOVA and Tukey's multiple comparison test. A probability (P) value of less than 0.05 was considered significant.

Results

The mice examined in this study showed significantly high serum enzyme activities of ALT and AST, 588.12 and 609.37 U/L, respectively (P<0.001, Figure 2), 4 h following the administration of 300 mg/kg of acetaminophen. All parameters significantly improved in *Epaltes* treated mice as compared to the acetaminophen control, post-treatment being better than pre-treatment. Percentage improvement of serum ALT, AST, and ALP were 51.82, 42.10, 48.20 and 54.76, 51.63, 77.81 in the pre- and post-treated groups, respectively (P<0.05). In the present study, the liver GSH concentration decreased significantly to 346.0 µg/g liver in the acetaminophen control group compared to 2916.04 µg/g liver in the control group (P<0.001, Figure 2). Both pre and post treatments with *Epaltes* increased GSH activity significantly (P<0.05) from 346.16 µg/g liver in the acetaminophen treated group to 1232.17 and 1388.44 µg/g liver in groups pre and post treated with *Epaltes*. In the NAC-treated group, there was a marked reduction in the serum enzyme concentrations and a significant increase in the GSH concentration. The percent reductions in serum ALT, AST, and ALP were 97.8, 94.9, and 46.98, respectively, whereas the percent increase in GSH concentration was 871.6 (P<0.05).



Effect of Epaltes and Paracetamol on serum enzyme levels of ALT, AST and ALP



Effect of Epaltes and Paracetamol on liver reduced glutathione level

Figure 2: 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 paracetamol (300 mg/kg in saline) and sacrificed 4h later; Group 4: Paracetamol + a single dose of N-acetyl cysteine (500 mg/kg) and sacrificed 4h later; Group 5: Post-treatment, sacrificed 4h later; Group 6: Pre-treatment, sacrificed 4h later. Results given as mean S.E.M. ALT-alanine aminotransferase, AST-aspartate aminotransferase, ALP-alkaline phosphatase. n=20 mice in each group.

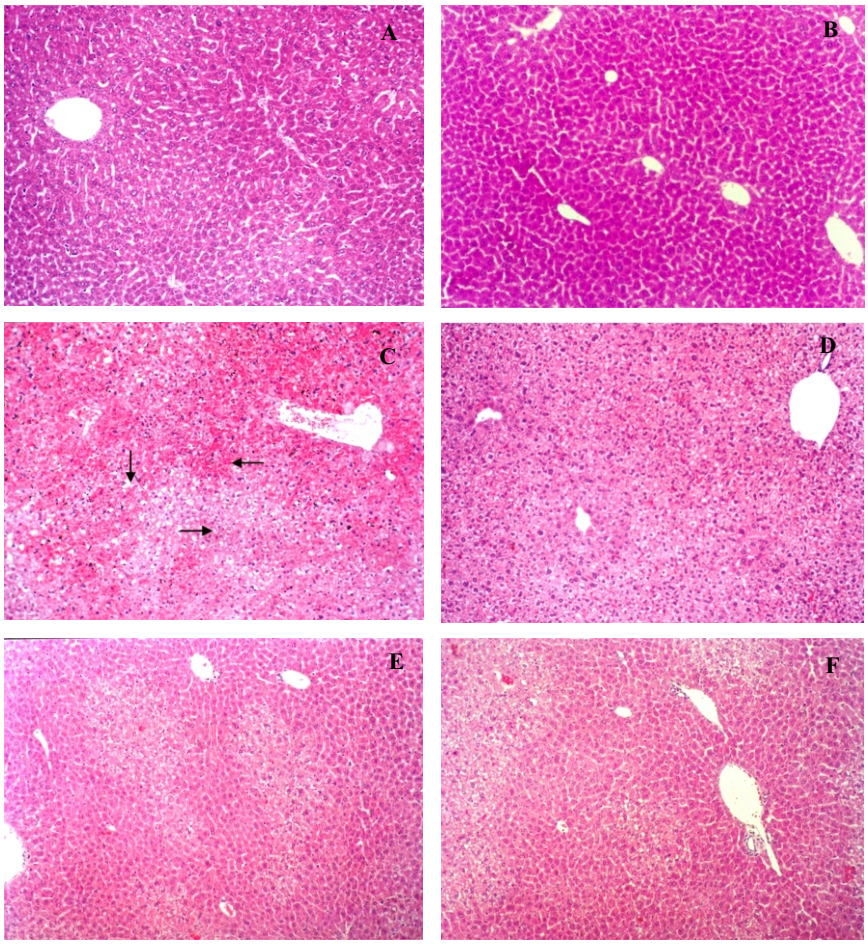


Figure 3: Assessment of acetaminophen induced hepatotoxicity by histopathology.

Haematoxylin and eosin stained liver sections. A: untreated; B: *Epaltes* (0.9 g/kg, p.o) for 7 days. C: acetaminophen control group shows severe confluent necrosis 4h after the administration of acetaminophen D: acetaminophen + N acetylcysteine 4h after the administration of acetaminophen does not show necrosis but vacuolar degeneration is visible. E: Post-treatment with *Epaltes* and sacrificed 4h later show necrosis (⟶), congestion (⟵), ballooning degeneration and vacuolation (⇓) F: Pre-treatment with *Epaltes* for 7 days and sacrificed 4h after the administration of acetaminophen shows no necrosis but diffused vacuolar degeneration is visible. 100 x magnification.

Histopathological Assessment of Liver Damage

Histopathological examination provided supportive evidence for the results obtained from the enzyme analysis. Microscopically, liver sections from control animals stained with haematoxylin and eosin showed normal parenchymal architecture with cords of hepatocytes, portal tracts, and centrilobular venules without noticeable alterations. Macroscopically, the liver appeared dark and congested in acetaminophen-treated mice. Histologically, the liver showed confluent necrosis with vacuolation and ballooning degeneration in the surviving hepatocytes (Figure 3C). NAC treated liver tissue did not show necrosis but vacuolar degeneration was visible. Compared to the acetaminophen control, extent of damage was less in the *Epaltes* post-treated group (Figure 3E), whereas in the pre-treated group, no significant change was noted (Figure 3F). No deviation from the normal parenchymal architecture was noted in the *Epaltes* control group (Figure 3B).

Histopathological observations clearly complement--ed results from biochemical analysis and overall results indicated that post-treatment of *Epaltes* is superior to pre-treatment in the model of acetaminophen induced hepatotoxicity.

Discussion

Acetaminophen is mainly metabolized by sulfation and glucuronidation at therapeutic doses. A small proportion is metabolized through cytochromes P450 2E1, 1A2, 3A4, and 2A6 to form a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) which binds covalently to proteins. Following therapeutic doses NAPQI is efficiently detoxified by conjugation with reduced glutathione (GSH). However, overdose causes depletion of GSH by as much as 90%, leading to the formation of acetaminophen protein adducts such as acetaminophen-cysteine adducts (13). The acetaminophen toxicity following NAPQI generation is chiefly due to oxidative stress and can effectively be ameliorated by antioxidants.

There are a number of natural drugs that have been claimed to have curative effects of liver disorders in traditional medicine. However, scientific information regarding the efficacy of many of the plants with reputed hepatoprotective activity is far from adequate. The purpose of this study was to evaluate the hepatoprotective effect of *Epaltes divaricata* compared to the known antidote NAC and to investigate its antioxidative properties as a mechanism of action. Initially, the hepatoprotective effect of the plant extract was identified by the determination of serum enzyme activities of ALT, AST, ALP, and histopathology. Significant increase in serum enzyme activity after the administration of acetaminophen in this study is consistent with previous data published by Ali, Bashir, and Rasheed (14) where increase in serum ALT and AST activities to 500.0 and 381.10 U/l were published in mice intoxicated with acetaminophen. All serum enzyme concentrations were significantly decreased in both *Epaltes* pre-treated and post-treated mice. The histopathological observations showing a faster regeneration of hepatic cells in mice suggest the possibility that the plant extract may possess the ability to condition the hepatic cells to a state of accelerated regeneration, thus decreasing the leakage of ALT, AST, and ALP into systemic circulation. This is consistent with previous findings by Singh and Handa (15) where the plants extract treated groups showed significant improvements in the liver histopathology as compared to the acetaminophen control group. Thus, from the results of the preliminary investigation it was concluded that the plant extract possesses a hepatoprotective effect against acetaminophen-induced hepatotoxicity in mice. Oxidative stress is a mechanism that has been postulated to be important in the development of acetaminophen toxicity. Accumulation of harmful oxidants in the cell as a consequence of oxidative stress is prevented through the action of small antioxidant molecules such as GSH, vitamins, and antioxidant enzymes (16). GSH is a critical determinant of tissue susceptibility to oxidative damage, and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals including acetaminophen and carbon tetrachloride (CCl_4) (17). Cell injury induced by xenobiotics occurs only if mitochondrial GSH is depleted. In the present study, the liver GSH level was decreased 88.1% in the acetaminophen treated group as compared to the control group ($P<0.001$). The results of the present study are in agreement with results reported by Lin et al. (18) where 130% decrease in liver GSH activity was observed as compared to the control group. Since the toxicity is enhanced by factors that cause GSH depletion,

enhanced NAPQI formation or reduction in the antioxidative capacity of the liver, it could be suggested that the partial hepatoprotection afforded by *Epaltes* may be ascribed to the opposing action on one or more of these factors. Increased GSH level in mice post-treated with plant extract may result from the enhancement of either de novo GSH synthesis or GSH regeneration or both. As a consequence of the action of plant extracts in GSH metabolism, hepatic GSH level can be sufficiently maintained to counteract the increased formation of free radicals as in the case of carbon tetrachloride toxicity.

A comparison of the hepatoprotective activity of the plant extract with NAC, the widely used antidote for acetaminophen poisoning, showed that under the experimental conditions used, plant extract was not as effective as NAC. Serum enzyme activities of ALT, AST, and ALP in NAC-treated mice were reduced by 97.8, 94.9, and 46.9%, respectively as compared to the acetaminophen-treated group. But percentage reductions of the same in *Epaltes*-treated mice were 54.76, 51.63, and 77.81%, respectively. There are limitations to the use of NAC. The effect of the treatment is extremely limited if it is not given within 16 hr of the overdose. Further- more, NAC is associated with a number of adverse reactions (19) such as nausea, diarrhoea, vomiting, rashes, and anaphylaxis (20).

Although the mechanism of chemical-induced liver injury is different from that of viral hepatitis, the pathological changes of parenchymal cell necrosis are a common phenomenon. Therefore, compounds that can either decrease the necrotic damage to hepatocytes via enhanced defense mechanisms against toxic insult or increase repair of damaged hepatocytes are considered potentially useful in the treatment of human hepatitis (21). Further studies are required to determine the active components of *Epaltes* and the exact mechanism that underlie its protective effect against liver damage. Based on the biochemical and histopathological evidence, it can be concluded that *Epaltes* has hepatoprotective and antioxidant activity against acetaminophen-induced hepatocellular damage in mice.

The observed protective effect of *Epaltes* against the hepatotoxin, acetaminophen, may be attributed to the presence of different phytochemicals. The flavonoids are known to be antioxidants, free radical scavengers and antiperoxidants leading to hepatoprotection. While the present investigation has scientifically confirmed the ability of the aqueous extract of *Epaltes divaricata* as an effective hepatoprotectant in mice model, further studies are needed to examine its effects on patients. Also its exact mechanism of action needs to be established.

Acknowledgements

The financial assistance provided by the National Science Foundation, Sri Lanka (Research Grant No: RG/2001/M/10), and the gift of acetaminophen by the Sri Lanka Pharmaceutical Manufacturing Corporation are gratefully acknowledged. We also thank Mrs. G.G.D.D. Gunawardane and Mr. D.G.P. Pathmabandu for the technical assistance provided.

References

1. Wojcikowski K, Gobe G. Animal studies on medicinal herbs: predictability, dose conversion and potential value. *Phytotherapy Research* 2014; **28**(1): 22-7.
2. Hewawasam RP, Jayatilaka KAPW, Pathirana C, Mudduwa LKB. Protective effect of *Asteracantha longifolia* extract in mouse liver injury induced by carbon tetrachloride and paracetamol. *Journal of Pharmacy and Pharmacology* 2003; **55**(10): 1413-1418.
3. Hewawasam RP, Jayatilaka KAPW, Pathirana C, Mudduwa LKB. Studies on the preventive and curative effects of *Epaltes divaricata* (Family-Compositae) extract on carbon tetrachloride induced hepatotoxicity in mice. *Indian Journal of Medical Research* 2004; **120**: 30-34.
4. Hewawasam RP, Jayatilaka KAPW, Pathirana C. Effect of *Asparagus falcatus* on acetaminophen toxicity in mice: A comparison of antioxidative effect with N-acetyl cysteine. *Journal of Dietary Supplements* 2008; **5**(1): 1-19.
5. Nelson SD. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. Seminar in Liver Disease 1990; **10**: 267-278.
6. Black M. Acetaminophen toxicity. *Annual Review of Medicine* 1984; **35**: 577-593.
7. Marx J. Protecting liver from painkiller's lethal dose. *Science* 2002; **298**: 341-342.
8. Eddleston M. Patterns and problems of deliberate self poisoning in the developing world. *Quarterly Journal of Medicine* 2000; **93**: 715-731.

9. James LP. McCullough SC. Lamps LW, Hinson JA. Effect of N-acetylcysteine on acetaminophen toxicity in mice: Relationship to reactive nitrogen and cytokine formation. *Toxicological Sciences* 2003; **75**: 458-467.

10. Dhawan BN, Srimal RC. Acute toxicity and gross effects. In Laboratory Manual for Pharmacological Evaluation of Natural Products. United Nations Industrial Development Organization and International Centre for Science and High Technology. Italy, 1998: 17-20.

11. Reitman S, Frankel S. A colorimetric method for the determination of serum levels of glutamic oxaloacetic acid and pyruvic acid transaminases. *American Journal of Clinical Pathology* 1957; **10**: 394-399.

12. Jollow DZ, Mitchel JR, Zampaglione N, Gillete JR. Bromobenzene induced liver necrosis: Protective role of glutathione and evidence for 3-4 bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology* 1974; **11**: 151-169.

13. James P, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. *Drug Metabolism and Disposition* 2003; **31**(12): 1499-1506.

14. Ali BH, Bashir AK, Rashid RA. Effect of the traditional medicinal plants *Rhazya stricta, Balanitis aegyptiaca* and *Haplophyllum tuberculatum* on paracetamol induced hepatotoxicity in mice. *Phytotherapy* Res 2001; **15**: 598-603.

15. Singh A, Handa SS. Hepatoprotective activity of *Apium graveolens* and *Hydrophila auriculata* against paracetamol and thioacetamide intoxication in rats. *Journal of Ethnopharmacology* 1995; **49**: 119-126.

16. Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicological Sciences* 2001; **59**: 169-177.

17. Ko K, Ip M, Poon SP, Wu MKT, Che SS, Ng CT, Kong YC. Effect of lignan enriched *Fructus schisandrae* extract on hepatic glutathione status in rats: Protection against carbon tetrachloride toxicity. *Planta Medica* 1995; **61**: 134-137.

18. Lin S, Chung T, Lin C, Ueng T, Lin Y, Lin S, Wang L. Hepatoprotective effects of *Arctium lappa* on carbon tetrachloride and acetaminophen- induced liver damage. *American Journal of Chinese Medicine* 2000; **28**(2): 163-173.

19. Sies H. Strategies of antioxidant defense. *European Journal of Biochemistry* 1993; **215**: 213-219.

20. Schmidt LE, Dalhoff K. Risk factors in the development of adverse reactions to *N*-acetylcysteine in patients with paracetamol poisoning. *British Journal of Clinical Pharmacology* 2001; **51**(1): 87-91.

21. Perrissoud D, Testa B. Hepatic pharmacology: Mechanisms of action and classification of antinarcotic hepatoprotective agents. *Trends in Pharmacological Sciences* 1982; **2**: 356-357.