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Spathodea campanulata Extract Attenuates Acetaminophen-Induced Hepatic Injury in Mice

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ABSTRACT

Spathodea campanulata, well known for its traditional medicinal uses was investigated for hepatoprotective activity against acetaminophen-induced hepatic damage in mice. Six groups of mice were pre-treated with 100, 300 and 625 mg/kg of the aqueous extract of *Spathodea campanulata* stem bark, N-acetyl cysteine (300 mg/kg; p.o) or distilled water for 5 days before they were intoxicated with a single dose of acetaminophen (600 mg/kg; p.o). Alanine aminotransferase, Aspartate aminotransferase and total protein levels were measured in serum, glutathione peroxidase, superoxide dismutase and total cytochrome P_{450} levels were measured in liver homogenate and liver histology was also observed on liver sections. Total liver cytochrome P_{450} levels in *Spathodea campanulata* extract, distilled water, ketoconazole or phenobarbital-treated animals were also measured. Significant hepatoprotection was obtained against liver damage induced by acetaminophen as evident from decreased serum levels of Aspartate transaminase, Alanine transaminase and increased levels of total protein in the combined acetaminophen and extract treated groups and the acetaminophen and N-acetylcysteine-treated groups compared to the acetaminophen only controls. The decrease in serum antioxidant enzymes; glutathione peroxidase and superoxide dismutase levels caused by acetaminophen was significantly reversed by the extract. The results correlated well with the histopathology of liver from treated and control animals. The extract also caused considerable inhibition of total CYP_{450}, the enzymes involved in the activation of acetaminophen. The present results indicate that *Spathodea campanulata* protects the liver against acetaminophen-induced hepatotoxicity by enhancing antioxidant protection capacity and interfering with the bio-activation of acetaminophen.

Keywords: Antioxidant-enzymes, cytochrome P450, hepatoprotection, transaminases.

INTRODUCTION

Liver injury from overdose of acetaminophen (APAP), a well prescribed analgesic and antipyretic [1] remains one of the most common causes of death worldwide. In the United States and the United Kingdom it is the most common cause of acute liver failure. [2] In most African countries where public health systems are considered relatively weak, acetaminophen-related liver diseases can be underestimated. The mechanisms of acetaminophen toxicity have been well studied. [3] Acetaminophen is activated by cytochrome P_{450} to form a reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). [4] Though this metabolite is highly reactive, it effectively combines with glutathione (GSH) in liver cells to form mercapturic acid which is soluble and is excreted by the kidneys. In excessive doses, NAPQI depletes GSH stores and binds covalently to macromolecules in the cell to generate reactive oxygen species and free radicals. [5] This chain of events results in oxidative stress which plays a central role in the hepatic damage caused by acetaminophen.

Antioxidants have been investigated as alternative treatment against acetaminophen toxicity. [6] One of such antioxidants is N-acetylcysteine (NAC) [7], which prevents N-acetyl-p-benzo-quinone imine (NAPQI) from binding to hepatic macromolecules in the early phase of acetaminophen intoxication (< 8 h). [8] It exerts its effect by acting as a glutathione precursor or alternatively as a sulfate precursor. [9] Upon deacetylation, NAC becomes L-cysteine, entering cells where it may serve as a precursor for GSH synthesis. Indeed, NAC has been shown to prevent GSH depletion and/or cause an increase in hepatic GSH levels.

NAC is now accepted as the mainstay in the management of acetaminophen over dosage but it is not readily accessible and affordable in most developing countries.

We observed in our laboratory that *Spathodea campanulata* known commonly as African Tulip tree stem bark used for a

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variety of disorders in Africa [10-11] showed promising hepatoprotective activity against carbon tetrachloride induced hepatotoxicity in rats (unpublished). In the present study, we report on the effect of the aqueous stem bark extract of *Spathodea campanulata* on acetaminophen-induced hepatic damage in mice.

MATERIALS AND METHODS

Chemicals/ Reagents

N-acetylcysteine (NAC), Tris-HCl, phosphate buffered saline (PBS), sodium deoxycholate, Triton X-100, Sodium dodecyl sulphate (SDS), Ethylene diamine tetra acetic acid (EDTA), Bovine Serum albumin (BSA), phenobarbital, ketoconazole, phenyl methane sulphonyl fluoride (PMSF), sodium dithionite, glutathione (GSH), β-NADPH, glutathione reductase, sodium azide, cytochrome c from horse heart, nitroblue tetrazolium chloride (NBT), xanthine oxidase, xanthine sodium salt, Glutathione peroxidase (GPx) and sucrose were purchased from Sigma (St. Louis, MO, U.S.A.). Acetaminophen powder was purchased from LETAP Pharmaceutical Company, Accra, Ghana, and hydrogen peroxide was obtained from Kama Health Services, Kumasi, Ghana.

Animals

The animals used in the study, male ICR mice, were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon and maintained in the Animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. The animals were housed in groups of 6 in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Preparation of *Spathodea campanulata* Extract

Spathodea campanulata stem bark was collected at Kotei, a suburb of Kumasi, near Kwame Nkrumah University of Science and Technology, Kumasi, Ghana in the month of October, 2008 and authenticated by Dr Kofi Annan, a Senior Pharmacognosist at the Department of Pharmacognosy, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi. The stem bark was cut into smaller pieces and sun dried for nine days. The dried stem bark was milled in a hammer mill. The powdered stem bark (1000 g) was then extracted by decoction in water (3 litres) for thirty minutes and the extract freeze-dried. The yield was 15.2 % w/w. The freeze-dried extract, referred to as SCE or the extract in this study was then stored in a desiccator and was reconstituted in water immediately prior to use.

Effect of the extract (SCE) on acetaminophen-induced hepatotoxicity

The effect of SCE on acetaminophen-induced hepatic damage was investigated according to the method described by Ali *et al.,* (2001). [5] Forty eight (48) male mice were placed in six groups (n = 8). Groups (1-4) received SCE (100, 300, 625 mg/kg; p.o) and N-acetyl cysteine (300 mg/kg; p.o) daily respectively for five consecutive days. Groups (5 and 6) were to serve as the negative and positive controls and received distilled water only over the 5-day period. Two hours after the last drug administration, all the groups, except group 5 received 600 mg/kg acetaminophen suspension in distilled water orally. Group 5 served as the negative control and received distilled water. Three hours after acetaminophen administration animals were euthanized. Blood samples were collected from a common carotid into dry centrifuge tubes. After blood collection, livers were removed for tissue studies. The coagulated blood samples in the plain tubes were tested for markers of liver damage. Liver slices fixed for 12 hours in Bouin's solution were processed for paraffin embedding following standard micro techniques. [12] Sections of liver (5µm) stained with alum haematoxylin and eosin was observed microscopically for histopathological changes.

Effect of SCE on liver Cytochrome P_{450} enzymes in Mice

The cytochrome P_{450} content was measured according to the method described by Choi, *et al.,* (2003). [13] ICR mice weighing 25-30 g were used for the experiment. Animals were divided into four groups (n = 8) and housed for five days before treatment.

Group (A); received distilled water and served as control. Groups (B, C and D) received SCE (625 mg/kg; p.o), ketoconazole (100 mg/kg; p.o) and phenobarbital (100 mg/kg; p.o) respectively daily for seven consecutive days. On the eighth day, the animals were euthanized. The livers were removed, blotted of blood and homogenized in a 0.25M sucrose solution at a ratio of 5 ml/g of tissue. PMSF was added to inactivate serine proteases and the homogenate was centrifuged at 600 g for 5 min. The supernatant was further centrifuged at 12000 g for 10 minutes, and the second supernatant further centrifuged at 100,000 g for 1 hour. The resultant pellets were suspended in 0.25M sucrose. Protein concentration was adjusted to 20 mg/ml using a modified Lowry method of protein assay. The CYP_{450} level was then determined by forming the CYP_{450}-CO complex in a CO chamber leaving a non-CO complex control outside the chamber. The difference in absorbance at 450 and 490 was measured in a microplate reader (Bio-Tek ELx 808)

CYP_{450} levels were calculated as $[P_{450}]_{(mM)} = (\Delta Apc - \Delta Ap) / 91$;

ΔApc = change in absorbance of the P450-CO complex

ΔAp = change in absorbance of the non-CO control

91 = extinction coefficient

Antioxidant enzyme activity

Mice were treated as described elsewhere in this paper under the effect of the extract (SCE) on acetaminophen-induced hepatotoxicity. The treated animals were euthanized and their livers removed. Excised livers were perfused with phosphate buffered saline (PBS) containing 0.16 mg/ml of heparin. They were then homogenized in cold isotonic buffer in a 10 ml/g buffer/ tissue ratio.

Glutathione peroxidase (GPx) activity

The GPx activity was measured as described by Lawrence and Burk (1976). [14] To obtain the post-nuclear fractions for the GPx activity measurements, the homogenate was centrifuged at 100000×g for 10 minutes. The supernatant was collected and stored at -80ºC until required. For a 2 ml assay, 1.26 ml of assay buffer (50 mM sodium phosphate, pH 7.0), 0.2 ml of 10 mM GSH, 0.2 ml of 2 mM NADPH and 0.02 ml of (100 U/ml) glutathione reductase were mixed in a cuvette.

Sodium azide (0.02 ml) was added to the content of the cuvette. For the blank, 0.2 ml homogenizing buffer was added to the mixture in the cuvette whiles 0.2 ml of clarified sample was added for the test sample. The content of the cuvette was mixed and allowed to equilibrate for 5 minutes.

Hydrogen peroxide (0.1 ml) was then added, quickly mixed to start the reaction and the change in absorbance was measured at 340 nm for 5 minutes using a Shimadzu UV mini-1240, UV-vis Spectrophotometer.

The decline in the absorbance at 340 nm monitors the consumption of NADPH in the following reaction catalyzed by glutathione reductase:

$$H^+ + GSSG + NADPH \longrightarrow 2GSH + NADP^+$$

This reaction is driven by the formation of GSSG, coupling the glutathione peroxidase reaction to the change in absorbance monitored in the assay.

The NADPH consumption rate is used to calculate the glutathione peroxidase activity in the coupled assay. $(Abs/min \div 6.22\times10^3) \div 1000 = mol\ NADPH\ consumed/min$

The value 6.22×10^3 is the optical density of a 1M solution of NADPH in a path length of 1cm. The divisor of 1000 corrects the NADPH consumption from per liter to the actual assay volume of per ml. The enzyme activity units value was then divided by the amount of protein in the assayed sample aliquot, expressed in mg, to yield the sample specific activity.

Superoxide dismutase activity assay

The SOD activity was assayed according to the method of McCord and Fridovich (1969). [15] The homogenized sample was centrifuged at 1000×g at 4ºC for 10 minutes to isolate the crude nuclear fractions. The supernatant was further centrifuged at 3000×g at 4ºC for 10 minutes, at 20000×g for 20 minutes and 144000×g for 90 minutes. The final supernatant which is the cytosolic fraction was stored for the assay of SOD.

The reaction cocktail was prepared by mixing 125 ml PBS (pH 7.8), 5 ml EDTA (10.7 mM), 5 ml cytochrome c (1.1 mM), 250 ml xanthine salt solution (0.108 mM) and 115 ml distilled water. The pH was then adjusted to 7.8 at 25ºC. Xanthine oxidase enzyme solution, at a concentration of 0.05 U/ml, was prepared in phosphate buffered saline just before use.

In a cuvette, 2.8 ml reaction cocktail was mixed with 0.2 ml distilled water and absorbance at 550 nm was read for five minutes. This served as the blank.

For the uninhibited reaction, 2.8 ml reaction cocktail was added to 0.1 ml distilled water and 0.1 ml xanthine oxidase enzyme solution. Again the absorbance for five minutes was read at 550 nm.

For the inhibited reaction, 2.8 ml reaction cocktail was added to 0.1 ml xanthine oxidase enzyme solution and 0.1 ml of the sample to be tested. The content was immediately mixed by inversion and the increase in absorbance at 550 nm was recorded for five minutes.

Calculations:

$$\% \text{ Inhibition} = \frac{\Delta A/min\ (Inhibited) - \Delta A/min\ (Uninhibited)}{\Delta A/min\ (Inhibited) - \Delta A/min\ (Blank)} \times 100$$

$$\text{Volume activity in units/ ml enzyme} = \frac{\% \text{ Inhibition} \times \text{dilution factor}}{50\ \% \times 0.1}$$

50% = inhibition of the rate of cytochrome c reduction

0.1 = volume (ml) of enzyme used

$$\text{Specific activity in units/ mg protein} = \frac{\text{Volume activity in units/ ml enzyme}}{\text{mg protein / ml enzyme sample}}$$

One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system using xanthine salt solution and xanthine oxidase at pH 7.8 at a temperature of 25ºC in a 3 ml reaction volume. The xanthine oxidase should produce an initial (uninhibited) ΔA of 0.025 ± 0.005 per minute.

Statistical Analysis

The results of the above experiments were expressed as mean ± SEM. Results were statistically analysed using one-way ANOVA, followed by the Newman-Keuls Multiple Comparison Test. $P<0.05$ was considered to be significant.

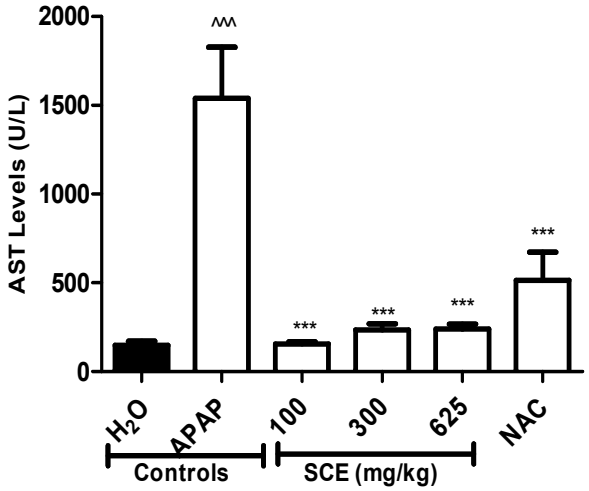

Fig. 1: Serum AST levels of mice pretreated with SCE before acetaminophen intoxication

Values are expressed as mean ± SEM (n=8). ^^^ indicates significance (p<0.001) compared to the normal control; * indicates significance (p<0.001) compared to the acetaminophen control**

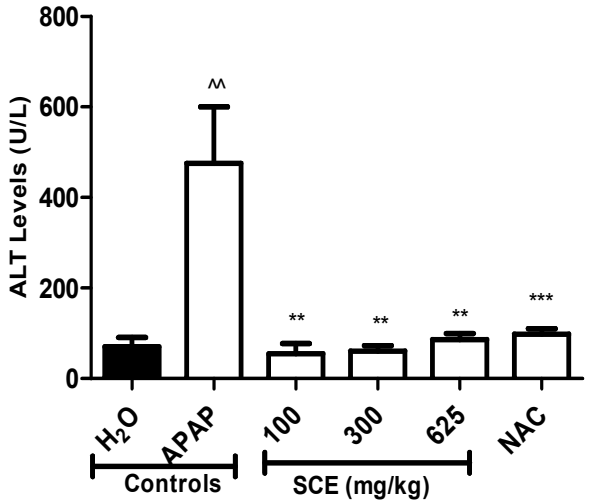

Fig. 2: Serum ALT levels of mice pretreated with SCE before acetaminophen intoxication

Values are expressed as mean ± SEM (n=8). ^^ indicates (p<0.01) compared to the normal control; ** indicates significance (p<0.01) compared to the acetaminophen control

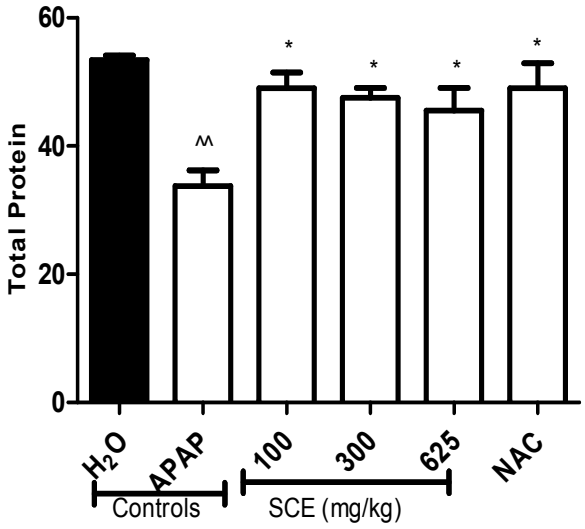

Fig. 3: Total protein in serum of mice pretreated with SCE before acetaminophen intoxication.
Values are expressed as mean ± SEM (n=8). ^^ indicates significance ($p<0.01$) compared to the normal control; * indicates significance ($p<0.05$) compared to the acetaminophen control

RESULTS

Effect of the extract (SCE) on acetaminophen-induced hepatotoxicity

The effect of SCE on acetaminophen-induced hepatotoxicity is observed in Fig. 1-4. The extract showed protective effect on the liver against acetaminophen-induced hepatotoxicity in mice. This was evident from the levels of serum AST, ALT and total protein (Fig. 1-3). Acetaminophen (600 mg/kg) increased the levels of the serum AST ($p< 0.001$) and ALT ($p<0.001$). These were reduced profoundly by pre-treatment with extract and N-acetyl cysteine ($p<0.001$ or $p<0.01$). Similarly, the decreased serum levels of total proteins ($p<0.01$) were restored by treatment at all doses of the extract and N-acetylcysteine ($p<0.05$).

The histopathology of mice treated with extract or NAC before acetaminophen intoxication is shown in figure 4. Liver sections taken from acetaminophen- treated mice had more inflammatory infiltration, hepatocyte coagulation and fibrous septa compared to controls. Histopathological analysis showed that NAC as well as the extract (625mg/kg) markedly improved the degree of hepatic fibrosis in acetaminophen-treated mice. Treated groups (APAP+ extract/NAC) displayed delicate fibrous septa, and lower collagen levels than the acetaminophen group (figure 4)

The 625 mg/kg dose suffered cellular degeneration, necrosis and inflammation to a lesser extent than the NAC treated group which suffered more inflammation and steatosis in zone 3 of the hepatocytes (Fig. 4).

Effect of extract on SOD and GPx activity

Acetaminophen also caused a profound decrease (three-fold less) in the SOD units. This was reversed by the extract and N-acetyl cysteine (Fig. 5).

Treatment of mice with acetaminophen significantly decreased GPx activity about (eight- fold less) compared to control ($p<0.001$). Treatment with SCE significantly restored the activity of GPx (Fig. 6) similar to the control and NAC group ($p<0.001$).

Effect of the extract on hepatic CYP_{450}

Seven days treatment of mice with extract resulted in a significant decrease in the total cytochrome CYP_{450} enzymes in the liver (Fig. 6 & 7). The decrease was comparable to ketoconazole, a known inhibitor of cytochrome CYP_{450}. Phenobarbital also increased the total cytochrome CYP_{450} enzymes compared to the control. Treatment of mice with acetaminophen resulted in up-regulation of the total CYP_{450} in the liver. The extract decreased the elevated total CYP_{450} but not NAC.

DISCUSSION

Acetaminophen is an extensively used antipyretic-analgesic drug which produces acute hepatic damage when taken in overdose. [16] It is now established that, a fraction of acetaminophen is converted via the cytochrome CYP_{450} pathway to a highly toxic metabolite; N-acetyl–p–benzoquinone amine (NAPQI) [17-18], which is normally conjugated with glutathione to be excreted in urine. Overdose of acetaminophen depletes glutathione stores through conjugation, leading to a build-up of NAPQI [7], which covalently binds with cellular macromolecules leading to hepatotoxicity. [19] Mitochondrial dysfunction and the development of acute hepatic necrosis are consequently potentiated.

In the present study, hepatic parenchymal cell injury was estimated by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum. It was established that a single dose of acetaminophen (600 mg/kg) caused injury to the parenchymal cells resulting in a leakage of the aminotransferases. These enzymes (ALT, AST) are released into circulation when there is hepatic necrosis making them measureable in serum. [20] High levels of AST could indicate liver damage, viral hepatitis, cardiac infarction and muscle injury. [21] ALT, is however a more specific marker for liver damage. [22] Significant elevation of AST and ALT indicates cellular leakage and loss of functional integrity of parenchymal cell in the liver. Consistent with these reports, administration of acetaminophen, a known hepatotoxin in over dosage caused a profound elevation in AST and ALT serum levels. Pretreatment of mice with the extract at all doses prevented the rise in the AST and ALT caused by acetaminophen. The results agrees with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes. [23]

The synthetic ability of the hepatocytes was estimated by a measurement of the total protein. Acetaminophen administration caused a significant decrease in total serum protein when compared to control. Pretreatment with the extract however attenuated significantly the damage to the parenchymal cells compared to the control group. This demonstrates clearly that the extract has the capacity to restore the synthetic ability of the liver which was perturbed by the administration of APAP. Extensive vascular degeneration and centrilobular necrosis was observed in the hepatocytes of animals treated with APAP. The livers of mice treated with APAP also showed gross vascular degeneration, steatosis and necrosis. This was prevented by pretreatment with SCE, evident by the absence or reduction of necrosis and vascular degeneration. The efficacy of a hepatoprotective agent is dependent on its ability to reduce the harmful effect or restore the altered hepatic physiology and histology caused by the hepatotoxin. Comparison of the

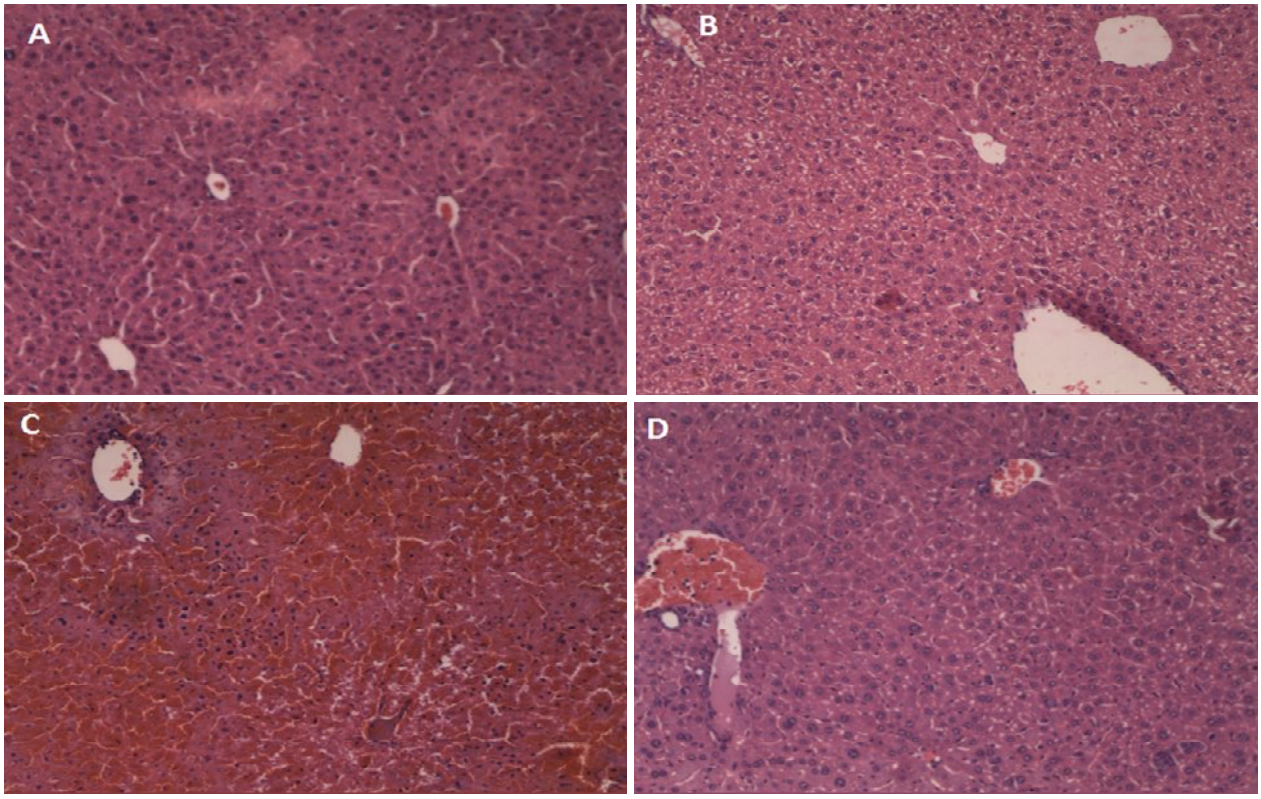

Fig. 4: Histopathological slides of livers of mice treated with acetaminophen after Spathodea or NAC treatment (40×). (A) Normal control, (B) Acetaminophen control, (C) NAC and (D) SCE – 625mg/kg

extract to NAC, the standard treatment for acetaminophen over dosage showed that the protective ability of SCE was similar to NAC.

The mechanism of hepatoprotection by *Spathodea campanulata* was also investigated in acetaminophen-induced acute liver injury model by assessing the possible antioxidant activity of SCE. An antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. [24] Antioxidants protect tissues against oxidative stress. Polyphenols act as antioxidants by scavenging reactive oxygen and nitrogen as well as chelating redox-active transition metal ions inhibiting them from catalyzing free radical formation. [25] Antioxidant enzymes such as SOD and GPx are also induced to scavenge reactive oxygen species generated by hepatotoxic agents. [26] Because partially reduced forms of oxygen during oxidation in a living system are cytotoxic, protective antioxidant enzymes including superoxide dismutase (SODs), catalase, and glutathione peroxidases usually exist to convert these reduced forms of oxygen to water. Superoxide dismutase is an enzyme which has an overall effect to lower the steady-state concentration of superoxide in the liver cells. [27] Pretreatment with SCE was able to obliterate the oxidative stress evident by the increase in SOD activity in the SCE treated groups similar to the control group.

SOD catalyzes the reaction:

$$O_2^- + O_2^- + 2H \rightarrow H_2O_2 + O_2$$

Using reduced glutathione as the reducing agent, glutathione peroxidases also convert hydrogen peroxide (H_2O_2) to water. Consequently, the oxidative stress is either reduced or eliminated. Hence, GSH constitutes the first direct line of defense against free radical activity in cells and is a critical determinant of tissue susceptibility to oxidative damage.

The test for glutathione peroxidase (GPx) activity confirmed that oxidative stress was induced by APAP. The decrease in GPx activity was restored by pretreatment with SCE.

Bio-activation has been postulated to play a critical role in hepatotoxicity of APAP. [28] APAP requires metabolic activation particularly by CYP450 enzymes to form reactive metabolites, which in turn cause liver injury both in experimental animals and humans. [29-31]

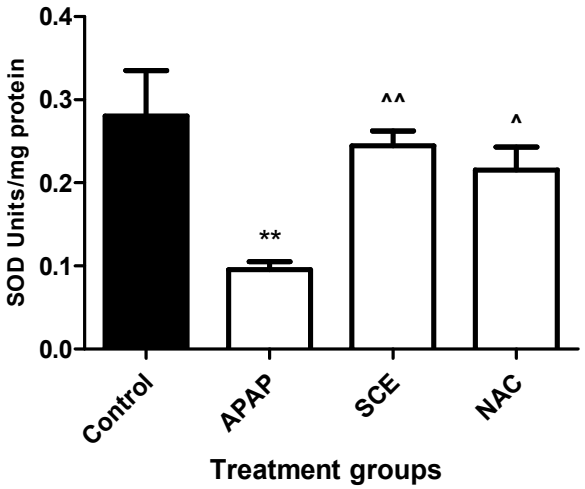

Fig. 5: Effect of SCE on SOD units of acetaminophen treated mice
Values are presented as the mean ± SEM (n=5). ** indicates significant decrease (p<0.01) compared to the control; ^^ indicates significance (p<0.01) and ^ indicates (p<0.05) compared to the acetaminophen group

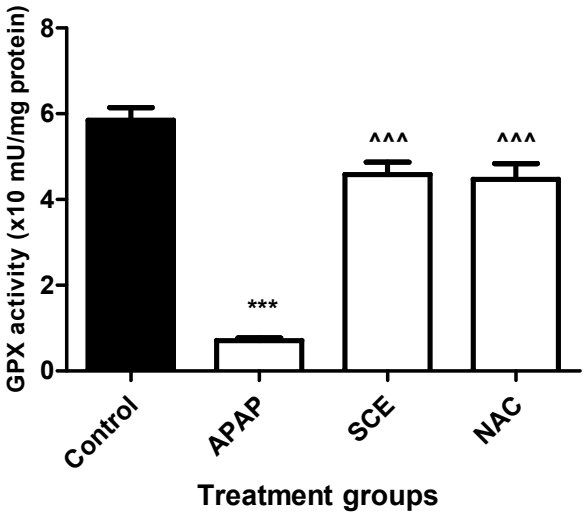

Fig. 6: Effect of SCE on glutathione peroxidase activity (GPx) of the livers of mice treated with acetaminophen
Values are presented as the mean ± SEM (n=8). * indicates significant decrease (p<0.001) compared to the control; and ^^^ indicates significant increase (p<0.001) compared to the acetaminophen treated control**

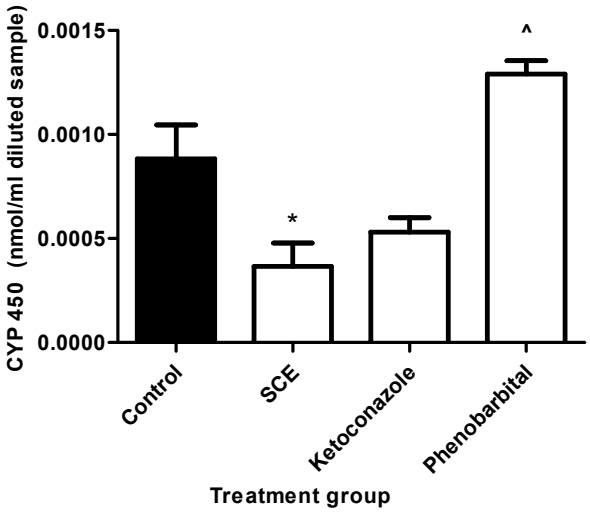

Fig. 7: Effect of SCE on cytochrome P450 content in the livers of mice
Values are presented as the mean ± SEM (n=8). * indicates significant decrease (p<0.05) compared to the control; and ^ indicates significant increase (p<0.05) compared to the control

In particular, APAP is metabolized by cytochrome P_{450} to NAPQI which is known to be both an oxidizing agent and an arylating agent. [7] NAPQI binds to cytosolic GSH and depletes it in the process. The major peroxide detoxification enzyme, GSH peroxidase, functions inefficiently under conditions of GSH depletion. [7] GPx therefore is expected to be inhibited when GSH has been depleted and *vice versa*. In addition, during formation of NAPQI by CYP_{450}, the superoxide anion is formed with dismutation leading to hydrogen peroxide formation. [29] Therefore, oxidative stress, the pivot of APAP toxicity to the liver originates from bio-activation. Consequently, total CYP_{450} content in the liver was assayed.

The total CYP_{450} levels in animals that received APAP alone remained significantly high. This agrees with the report that acetaminophen up-regulates CYP_{450}. [28] Treatment with the extract resulted in a decrease in the total CYP_{450} enzymes similar to ketoconazole. [32] The extent of acetaminophen-induced hepatotoxicity is reduced in the presence of enzyme inhibitors such as ketoconazole and isoniazid [32] in agreement with our present observation of reduction in APAP toxicity in the presence of the extract.

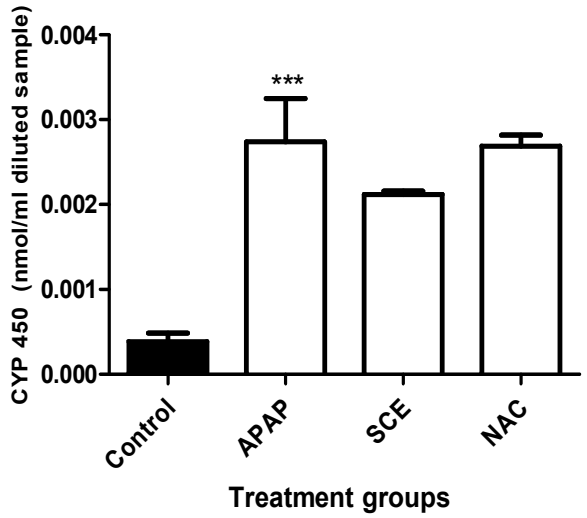

Fig. 8: Effect of SCE on total cytochrome P450 in the presence of acetaminophen. Values are presented as the mean ± SEM (n=8). * indicates significant increase (p<0.001) compared to the control**

Overall, *Spathodea campanulata* stem bark extract exhibits hepatoprotection against acetaminophen-induced hepatic damage by enhancing the levels of liver antioxidant enzymes and inhibiting CYP_{450} activity.

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