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Paracetamol Hepatotoxicity in Rats Treated With Crude Extract of *Alpinia galanga*

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Abstract: This study was conducted to observe the hepatoprotective effect of the crude extract of *Alpinia galanga* at 200 and 400 mg kg⁻¹ against paracetamol induced hepatotoxicity in rats. Forty male Sprague-Dawley rats were divided into groups of four consisting of a control, 3000 mg kg⁻¹ paracetamol as well as 200 and 400 mg kg⁻¹ *Alpinia galanga*. The control group was orally fed with distilled water for eight days while the 3000 mg kg⁻¹ paracetamol group was fed with distilled water for seven days followed by 3000 mg kg⁻¹ paracetamol on day eight. The extract of *Alpinia galanga* was fed for seven days based on the respective doses followed by 3000 mg kg⁻¹ paracetamol on day eight. Blood and liver samples were obtained from all the animals on the ninth day for biochemical analysis that includes total protein, aminotransferase enzymes (AST and ALT), malondialdehyde (MDA) and superoxide dismutase (SOD) as well as histological analysis (H and E staining). The results obtained showed that paracetamol given at the dose of 3000 mg kg⁻¹ induced hepatotoxicity with significant decrease in serum protein levels and significant increase in serum AST and ALT levels as well as liver MDA levels at p<0.05. Supplementation with the extract of *Alpinia galanga* maintained serum protein and liver SOD levels similar to that of the normal control group. Significant decrease (p<0.05) in liver MDA levels as compared with the group treated with 3000 mg kg⁻¹ paracetamol was observed in groups treated with the extract. Significant changes in MDA levels was also noted in group treated with 400 mg kg⁻¹ *Alpinia galanga* against the group treated with 200 mg kg⁻¹ *Alpinia galanga*. Histological analysis showed significant reduction in number of necrotic cells in both groups supplemented with the extract at p<0.05. The findings from the study showed that the crude extract of *Alpinia galanga* has protective effects against paracetamol induced hepatotoxicity.

Key words: Paracetamol, aminotransferase enzymes, malondialdehyde, superoxide, dismutase

INTRODUCTION

Paracetamol (PCM) or acetaminophen was discovered in Germany at the end of the 19th century, but was not widely used until mid way through the 20th century. PCM is probably the most versatile and widely used analgesic and antipyretic drug worldwide (Rocha *et al.*, 2005). Its potential hepatotoxicity was not suspected until the first clinical reports of severe and fatal liver damage following over dosage was reported by Davidson and Eastham (1966). PCM taken in over doses results in hepatotoxicity and nephrotoxicity in men and in experimental animal (Vermeulen *et al.*, 1992).

The chemical structure of PCM is N-acetyl-p-aminophenol (APAP). It has an excellent safety profile in therapeutic doses, but hepatotoxicity can develop with overdoses. The major target organ in PCM poisoning is the liver and the primary lesion is acute centrilobular

hepatic necrosis. In adults the single acute threshold dose for severe liver damage is 150 to 250 mg kg⁻¹ but there is marked individual variation in susceptibility. When taken in over dose it causes the production of reactive metabolite N-acetyl p-bezoquinonimine (NAPQI). Hepatotoxicity is the result of formation of the reactive and toxic metabolite NAPQI by the cytochrome P-450 system that covalently binds to cellular macromolecules and initiates cell damage (Vermeulen *et al.*, 1992) leading to glutathione depletion that increases liver susceptibility to oxidative stress (Cohen and Khairallah, 1997).

An important factor for avoiding complications in PCM poisoning is early administration of the antidotes, however with the emerging scientific researches on herbal medicines together with the global trend of increasing consumption of natural products, a naturally presenting antidote may already be present in the human

system through dietary intake to converse a protective effect against drug toxicity.

The use of the medicinal herbs for curing disease has been documented in history of all civilizations. According to the World Health Organization, 80% of the world population uses plant-based remedies as their primary form of healthcare (Evan, 1998). The use of herbal medicines in an evidence-or science-based approach to the treatment and prevention of disease is known as phytotherapy (Joanne *et al.*, 2002) flourishing the quest for significant source of synthetic and herbal drugs.

Alpinia galanga (*A. galanga*) or greater galangal locally known as lengkuas is a member of the Zingiberaceae (ginger) family. The ginger-like rootstock (rhizome) of the plant is widely used in Indonesia and Malaysia as a food flavouring and spice. The rhizome contains up to 1.5% essential oil which includes 1,8 cineol, α -pinene, eugenol, camphor, methyl cinnamate and sesquiterpenes. Resembling ginger in its effects, galanga is an aromatic stimulant, carminative and stomachic. It is used against nausea, flatulence, dyspepsia, rheumatism, catarrh and enteritis. It also possesses tonic and antibacterial qualities and is used for these properties in veterinary and homeopathic medicine.

This research is aimed to study the protective effect of the crude extract of *A. galanga* against PCM hepatotoxicity. The plants protective effect was assessed by monitoring liver function (serum protein, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), lipid peroxidation (hepatic malondialdehyde (MDA)) and endogenous antioxidant (superoxide dismutase (SOD)).

MATERIALS AND METHODS

Plant extraction: The rhizomes of *A. galanga* were cleaned and chopped into smaller slices. The sliced pieces weighing 2.8 kg were then mashed using a mortar and pestle and filtered to produce the crude extract. The crude extract was then freeze dried at -80°C to produce powdered extracts which were used for dose dependent preparation. Powdered extract were diluted in distilled water prior to administration.

Animal preparation and treatment regime: Forty male Sprague-Dawley rats (200-250 g) were obtained from the Animal House of Universiti Kebangsaan Malaysia. They were maintained on standard pellet diet and tap water *ad libitum*. The animals were kept in plastic cages under 12 h light/dark cycle. They were acclimatized to the environment for a week prior to experimental use.

The study design follows that of Jafri *et al.* (1999). PCM solutions were freshly prepared prior to administration by diluting soluble PCM in distilled water at a ratio of 1:2. The first group is used as a control that was fed orally with distilled water for eight days. The rats of the second group (PCM) were orally given distilled water for seven days and 3000 mg kg^{-1} of PCM orally to induce hepatotoxicity on the eight day. Rats of the third (200 AG + PCM) and fourth (400 AG + PCM) group were given an oral dose of *A. galanga* extract at concentrations of 200 mg kg^{-1} and 400 mg kg^{-1} , respectively for seven days followed by 3000 mg kg^{-1} of PCM on the eight day. On the ninth day of the study blood samples were obtained through cardiac puncture and the animals from all four groups were sacrificed to obtain liver samples for biochemical and histological studies.

Samples preparation: Blood samples were obtained through cardiac puncture from all animals used in the study prior to be sacrificed for the determination of serum total protein and enzyme activities of ALT and AST. Following sacrifice, the same lobes from the livers of the animals were preserved in 10% formalin for histopathological studies. For biochemical analysis, other parts of the liver were removed and homogenized in PBS buffer solution (pH 7.4) in a ratio of 4 mL g^{-1} (v/w) for lipid peroxidation product MDA and SOD enzyme activity determination.

Histopathology and biochemical assays: Serum total protein and liver enzyme activities of ALT and AST were determined using commercial kit using Bayer[®] clinical method for ADVIA[®] 1650 according to their instruction manual. The level of lipid peroxidation was determined by assessing the MDA concentration present in the sample. The assay was performed manually using the principle of adduct formation between TBA and MDA as a product of lipid peroxidation. The reaction yields a red MDA-TBA adduct, which forms a pink complex with absorption maximum at 532 nm.

The activity of SOD enzyme in liver homogenate was determined based on the activation of riboflavin by a proton which further oxidizes an electron donor which is the L-Methionine or EDTA. Oxidized L-Methionine or EDTA further reduces the riboflavin to a semiquinone state that leads to the reduction of oxygen to O_2^- which further reacts with nitroblue tetrazolium to form a purple colour. The chromogen formed is

then measured spectrophotometrically with a maximum absorbance at 560 nm.

For the histopathological examination, pieces of liver were fixed in 10% formalin and the hydrated tissue sections 5 µm thickness were stained with Hematoxylin and Eosin. The sections were then examined and scored under a light microscope.

Statistical analysis: The data are expressed as group Means±SEM. Levene's normality test was conducted prior to One-Way Analysis of Variance (ANOVA) with SPSS (version 12). ANOVA was performed to detect differences between all various groups. Significant differences detected by ANOVA were further analyzed using Tukey test. Values of p<0.05 were noted as significant.

RESULTS

Damage of liver is commonly assessed by determination of serum protein and serum aminotransferases (ALT and AST) activities. Administration of PCM reduced serum protein levels significantly (p<0.05) in the group treated with 3000 mg kg⁻¹ PCM with a mean value of 46.32±3.02 g L⁻¹ (Table 1) as compared with control group (58.33±1.66 g L⁻¹). However, supplementation with the extract of *A. galanga* at both doses did not show reduction in serum protein levels after 3000 mg kg⁻¹ PCM administration.

Significant increase in aminotransferase (ALT and AST) activities were observed in all three groups given an overdose of PCM compared to the control group (Table 1). Both groups supplemented with the extract however showed a slight reduction in aminotransferase activities compared to the group given 3000 mg kg⁻¹ PCM.

Table 1: Effects of PCM toxicity and extract administration on serum total protein, AST and ALT activities

Groups	Total serum protein (g L ⁻¹)	AST (IU L ⁻¹)	ALT (IU L ⁻¹)
Control	58.33±4.69	140.50±7.44	58.79±3.03
3000 mg kg ⁻¹ paracetamol	46.32±7.41**	272.33±24.46*	110.65±12/08*
200 mg kg ⁻¹ <i>Alpinia galanga</i> + paracetamol	58.77±4.12	232.10±22.8*	94.99±4.37*
400 mg kg ⁻¹ <i>Alpinia galanga</i> + paracetamol	59.68±2.92	209.70±11.5*	94.05±6.62*
3000 mg kg ⁻¹ <i>Alpinia galanga</i> + paracetamol			

Values are expressed as Mean±SD of 10 animals in each group. *Significantly increased against control group at p<0.05. **Significantly decreased against all other groups at p<0.05

The effects of *A. galanga* on PCM toxicity that induces lipid peroxidation were examined through monitoring the levels of MDA and SOD enzyme activity. Hepatic MDA shows significant increase (p<0.05) with a mean value of 3.79±0.33 nmol g⁻¹ in the PCM group (Table 2) compared to the control group (1.74±0.12 nmol g⁻¹). Hepatic MDA levels in the 200 AG + PCM group (2.52±0.27 nmol g⁻¹) showed significant increase as compared with control group and significant decrease as compared with PCM group. However, supplementation with 400 mg kg⁻¹ of extract lowered hepatic MDA levels (1.47±0.13 nmol g⁻¹) significantly as compared to the group given 200 mg kg⁻¹ of extract and the group given 3000 mg kg⁻¹ PCM only. SOD activities was found to be depleted in the group given 3000 mg kg⁻¹ PCM only as compared with the other groups in the study. Although the activity of SOD in the groups supplemented with the extract was not statistically different from the control group and the group given PCM alone, supplementation with the extract seem to reduce SOD depletion as shown in Table 2.

Histopathological analysis showed that the highest number of necrotic cells were observed in the PCM group with a mean value of 35.3±4.41. This value was found to be significantly increased compared to the control group (6.17±1.47) and the groups given the extract of *A. galanga* at 200 and 400 mg kg⁻¹ with mean values of 27.0±3.85 and 23.0±4.20, respectively (Table 3). The number of necrotic cells observed in the groups supplemented with the extract was found to be significantly increased against the control group but significantly decreased compared to the PCM group. Hepatocytes in the PCM treated group displayed clear cellular degeneration and loss of distinct lever characteristic. However, hepatocytes from the groups supplemented with the extract showed varying degrees of cellular degeneration with better preservation

Table 2: Effects of PCM toxicity and extract administration on hepatic MDA and SOD levels

Groups	MDA (nmol g ⁻¹)	SOD (u.e/min/g protein)
Control	1.74±0.37	101.85±9.74
3000 mg kg ⁻¹ paracetamol	3.79±0.82*	84.09±14.21
200 mg kg ⁻¹ <i>Alpinia galanga</i> + paracetamol	2.52±0.71**	96.49±9.63
400 mg kg ⁻¹ <i>Alpinia galanga</i> + paracetamol	1.47±0.41***	99.87±16.9
3000 mg kg ⁻¹ <i>Alpinia galanga</i> + paracetamol		

Values are expressed as Mean±SD of 10 animals in each group. *Significantly different from all groups at p<0.05. **Significantly different from control, PCM and 400AG + PCM groups at p<0.05. ***Significantly different from PCM and 200AG + PCM groups at p<0.05

Table 3: Effects of PCM toxicity and extract administration on liver cell necrosis

Groups	No. of necrotic cells
Control	6.17±1.47
3000 mg kg ⁻¹ paracetamol	35.32±4.41*
200 mg kg ⁻¹ <i>Alpinia galanga</i> + 3000 mg kg ⁻¹ paracetamol	27.01±3.85**
400 mg kg ⁻¹ <i>Alpinia galanga</i> + 3000 mg kg ⁻¹ paracetamol	23.04±4.2**

Values are expressed as Mean±SD of 10 animals in each group. *Significantly different from all groups at p<0.05. ** Significantly different from PCM group at p<0.05

of hepatic cell architecture. Higher dose of extract proved to have better prevention of necrotic changes with liver cells appearing almost normal except for a few scattered degenerated cells.

DISCUSSION

Paracetamol (Acetaminophen) is widely used as an analgesic and anti-pyretic throughout the world. Acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite (NAPQI) that covalently binds to protein (Mitchell *et al.*, 1973; Laura *et al.*, 2003). NAPQI is detoxified by glutathione (GSH) to form an acetaminophen-GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90% and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts (Mitchell *et al.*, 1973).

Administration of 3000 mg kg⁻¹ of PCM (Jafri *et al.*, 1999) in a single oral dose has proved to induce hepatotoxicity in the experimental animals. This is supported by the biochemical (serum: protein, ALT and AST; liver: MDA and SOD) as well as histological findings (number of necrotic cells) that showed a significant increase in the group compared against the control group. Hepatotoxicity was also induced in the groups treated with the extract of *A. galanga* as noted with biochemical as well as histological changes with a varying degree of severity. Supplementation with *A. galanga* reduces the severity of protein disruption as observed with the maintaining of protein concentration almost similar to the normal group as compared to groups supplemented with the extract prior to an overdose of PCM.

Reduction of protein concentration in paracetamol overdose may be due to the formation of protein adducts. Primary cellular targets have been postulated to be mitochondrial proteins, with resulting loss of energy production, as well as proteins involved in cellular ion control (Nelson, 1990). Formation of reactive NAPQI metabolite results in covalent modification of cellular

target proteins, cell death and organ damage (Zaher *et al.*, 1998). In a study by Qiu *et al.* (1998), matrix-assisted laser desorption ionization mass spectrometry was used to identify 20 proteins containing covalently bound acetaminophen. Covalent binding of NAPQI to proteins may induce denaturation of protein as well as changes in protein structures.

Though liver plays an important role in protein synthesis, changes in protein concentration is likely to occur in an acute liver disorder. Thus it can be concluded that reduction in protein concentration in animals treated with PCM may be due to disruption in protein structures and formation of protein adducts with the reactive metabolite (NAPQI) and also the possible involvement of nephrotoxicity that caused loss of protein through the renal route.

In the assessment of liver damage by PCM overdose the determination of enzyme levels such as AST and ALT is largely used as the most common biochemical markers of hepatocellular necrosis. High doses of paracetamol have been demonstrated to increase the serum levels of AST and ALT (Kozar *et al.*, 2003). High levels of ALT indicates liver damage with the highest increases (often >20 fold) being observed with acute hepatocellular injuries, such as xenobiotic-induced necrosis (Clermont *et al.*, 1967). ALT is highest in the liver and therefore, it appears to be a more sensitive test for hepatocellular damage (Shyamal *et al.*, 2006).

From this study, a significant increase in serum AST and ALT was noted in the group given 3000 mg kg⁻¹ of PCM alone. Increase in serum liver enzyme activity in this group is confirmed with the histopathological findings that indicate an increase in the necrotic cells. Increased free radical formation NAPQI in PCM overdose causes damage to cells and eventually necrosis that leads to leakage of these enzymes into the blood. Treatment with extract of *A. galanga* at a dose of 200 and 400 mg kg⁻¹ proved to reduce liver enzyme activity. The changes can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. This is also supported by the histological changes that show less severe cell damage in livers of the treated animals. Reduction in liver enzyme activity in the treated group however was not significantly reduced compared against the control group. However, it is known that liver enzyme activity concentration changes in a span of 24 to 72 h after damage has occurred. It is also known that the degree of increase in the enzyme activity does not correlate well with the extent of liver injury or prognosis. A sequential

monitoring of the liver enzyme activity would be needed for better assessment.

Another possible marker of liver injury includes increase in oxidative stress as evident by lipid peroxidation and reduced antioxidant enzymes. Earlier studies on PCM toxicity has indicated that formation of reactive metabolite NAPQI has a cause of increased lipid peroxidation that has been postulated as a destructive process in liver injury due to PCM administration (Muriel *et al.*, 1992). The increase in MDA levels is the most frequently used indicator of lipid peroxidation (Flemming *et al.*, 1997) and suggests enhanced free radical insult leading to tissue damage and failure of antioxidant defense mechanisms (Kanda *et al.*, 2005).

From the experiment, high level of MDA was found in the group given 3000 mg kg⁻¹ of PCM alone. This increase is noted to be significant compared against the other groups in the study. Supplementation with the extract showed to lower lipid peroxidation as noted with the significantly lower levels of MDA in the groups. Treatment with 400 mg kg⁻¹ of extract also showed a significantly lower MDA levels compared with that was given 200 mg kg⁻¹ of the extract. *A. galanga* is a plant that is known to have high levels of antioxidant properties. A study by Padma *et al.* (2004) on antioxidant screening study of Zingiberaceae family found that methanolic extract of *A. galanga* to be the most promising plant with highest antioxidant properties noted. Supplement of 7 days with the extract of the plant has shown to have protective effect against lipid peroxidation in particular as observed from the study.

Antioxidant enzymes such as SOD are known to reduce the harmful effects of free radicals (Topic *et al.*, 2002) and have been reported as one of the most important enzymes in the enzymatic antioxidant. Reduction in antioxidant defense system eventually leads to lipid peroxidation, protein and DNA damage (Diana *et al.*, 2002). Increase in serum activity of SOD is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury (Curtis *et al.*, 1972). From the experiment no significant changes were observed in liver SOD activity of the animals. However, liver SOD activity in the group given PCM alone was found to be depleted compared against the other groups in the study. Groups treated with the extract showed an improved SOD activity as noted by the increased levels of the enzyme activity. Although the results obtained showed no significant changes among the groups, increased SOD activities in the group supplemented with the extract shows a potential protective effect of the plant against oxidative damage

to the tissues and improvement in hepatic antioxidant enzyme system. Phenolic compounds such as flavanoid present in the extract acts as a potential precursor of superoxide anions as well as a potential antioxidant (Benevente, 1997). It is known that the metabolic pathway of PCM consumes the antioxidant glutathione peroxidase to a greater extend then the SOD. Improvement in SOD activities, however, does provide evidence to an overall improvement in endogenous antioxidant defense system. This relates to the fact that *A. galanga* has been shown to posses high antioxidative activity and acts as a antioxidant that reduces free radical activity (Juntachote and Berghofer, 2005).

Changes in biochemical markers relate to the histopathological changes observed from the liver section of the animals in the study groups. Morphology of necrosis is noted by eosinophilic (pink), glassy and vacuolated cells with fragmented membrane. Liver samples of PCM treated group showed gross necrosis of the centrilobular hepatocytes characterized by nuclear pyknosis, karyolysis and eosinophilic infiltration. These findings were found 24 h post administration and were also found to be significantly higher in number of necrotic cells compared against the other groups in the study. Histopathological changes of groups treated with the extract showed a significant improvement in architecture. Though necrotic changes were still evident, the severity of the damage was less intense significantly. Treatment with 400 mg kg⁻¹ of the extract proved even better improvement in cell architecture with less necrotic cells and disruption to overall histological features.

From the study it can be concluded that the crude extract of *A. galanga* has promising hepatoprotective effect as observed through the improvement in the biochemical parameters as well as histological evidence. The plants high antioxidative property is likely to reduce cellular damage due to PCM toxicity.

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