

Therapeutic efficacy of *Acorus calamus* on acetaminophen induced nephrotoxicity and oxidative stress in male albino rats

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Abstract

Acorus calamus (AC) is a traditional medicinal plant that is commonly used for disorder of central nervous system abnormalities. In ayurvedic medicine, it is used for the treatment of insomnia, melancholia, epilepsy, hysteria, loss of memory remittent fevers and neurosis. This plant extract is mainly used for various pharmacological activities like antidiabetic, antiproliferative, immunosuppressive, antidiarrhoeal and hypolipidemic activities. The main constituents of AC were found belonging to monoterpene, sesquiterpene, phenylpropanoid, flavonoid and quinone. The aim of this study was to investigate the nephroprotective and antioxidant activities of ethanol extract of AC at two dose levels of 250 and 500 mg/kg B/W on acetaminophen (APAP) induced toxicity in male albino rats. APAP significantly increased levels of serum urea, hemoglobin (Hb), total leukocyte count, packed cell volume, creatinine, DLC, and mean corpuscular volume, raised body weight, and reduced levels of neutrophils, mean corpuscular Hb content, mean corpuscular hematocrit, granulocytes, uric acid, and platelet Concentration. AC inhibited the hematological effects of APAP. AC significantly increased activities of renal superoxide dismutase, catalase, glutathione, and glutathione peroxidase and decreased malondialdehyde content of APAP-treated rats. Apart from these, histopathological changes also showed the protective nature of the AC extract against APAP induced necrotic damage of renal tissues. In conclusion it was observed that the ethanol extract of AC conferred nephroprotective and antioxidant activities by histopathological and biochemical observations against APAP induced renal damage in rats.

Key words: *Acorus calamus*, nephrotoxicity, oxidative stress, acetaminophen.

Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is safely employed for a wide range of treatments (Yapar et al. 2007), overdose of APAP in human is fairly common and is often associated with hepatic (Nelson 1995, Boelsterli 1993, Holtzman 1995) and renal damage (Placke et al. 1987, Trumper et al. 1998, Ghosh et al. 2007). Although nephrotoxicity is less common than hepatotoxicity in APAP overdose, renal tubular damage and acute renal failure can occur even in the absence of liver injury (Carpenter et al. 1981, Jones et al. 1993, Eguia et al. 1977) and can even lead to death in humans and experimental animals (Ray et al. 1996, Webster et al. 1996). Studies are going on throughout the world for the search of protective molecules that would provide maximum protection to the liver, kidney as well as other organs and practically very little or no side effects would be exerted during their function in the body (Montilla et al. 2005, Mansour et al. 2006).

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A number of herbs are traditionally used in different countries in response to drug or toxin induced hepatic and renal disorders (El-Beshbishy et al. 2005). *Acorus calamus L* also known as sweet flag is a native plant of India. It is commonly known as Bach or Uragandha in north India. It is a semi aquatic, perennial, aromatic herb with creeping rhizomes. It exhibits polyploidy. This plant belongs to araceae family and has been used in the Indian and Chinese system of medicine for hundreds of years to cure disease especially the CNS abnormalities (Mukherjee et al. 2007, Lai et al. 2002, Shukla et al. 2006, Koo et al. 2003).

Ethanol extract of this plant traditionally used for antidiabetes (Cesspooch 2005, Letitia et al. 2002), antiproliferative and immunosuppressive (Mehrotra et al. 2003), anti diarrhoeal (Shoba and Thomas 2001), and hypolipidemic (Parab and Mengi 2002) activities. It is reportedly useful in clearing speech to the childrens (Ignacimuthu et al. 2006, Chellaiah et al. 2006) and allopathic (Nawamaki et al. 1996) properties.

In ayurvedic medicine it is used for the treatment of Insomnia, Melancholia, epilepsy, hysteria, loss of memory remittent fevers (Agarwal et al. 1956) and neurosis (Shukla et al. 2001, Agarwal et al. 1956). Recently, *Acorus calamus* proved high antioxidant activity (Acuna et al. 2002, Shahin Sharif Ali et al. 2008). The main constituents of AC were found belonging to monoterpene, sesquiterpene, phenylpropanoid, flavonoid and quinone (Patra and Mitra 1979).

The rhizome contains active ingredients possessing insecticidal (Singh and Upadhyay 1993, Schmidt and Streloke 1994, Perrett and Whitfield 1995, Sugimoto et al. 1995, Paneru et al. 1997, Rham and Schmidt 1999, Raina et al. 2003, Lahlou 2004), anti fungal (Lee et al. 2004), antibacterial (Mc graw et al. 2002), mitogenic activity towards human lymphocytes (Jagmoha et al. 2005).

An earlier study showing that the essential oil from this plant is b-asarone which is demonstrated to be responsible for anti carcinogenic activity (Hu et al. 1986, Taylor et al. 1967) as well as anti proliferative, immunosuppressive (Mehrotra et al. 2003), Sedaative and hypothermic effects (Zanoli et al. 1998). The sweet flag is an important medhya drug, capable of improving memory power and intellect. It is used in vitiated conditions of vata and kapha, stomatopathy, hoarseness, colic, flatulence, dyspepsia, helminthiasis, amenorrhoea, dismenorrhoea, nephropathy, calculi, strangury (Joy et al. 1998).

However, the nephroprotective effects of this plant extract have not been shown in scientific research work. Keeping this in view the present study is aimed to evaluate the nephroprotective and antioxidant activities of ethanol extract of *Acorus calamus* against APAP induced toxicity in rats.

Materials and Methods

Plant material: Aerial part of *Acorus calamus* (Araceae) was collected from Tirunelveli district, Tamil Nadu, India in the month of March. The plant material was taxonomically identified and authenticated by V.Chelladurai (Research Officer) Botany (C.C.R.A.S) Government of India. Voucher specimen (AECBT-07/2007-2008) has been retained in the Anna bioresearch foundation, Arunai engineering college, Tiruvannamalai, Tamilnadu, India.

Extraction: The aerial part of *Acorus calamus* was dried under shade and then powdered with a mechanical grinder to obtain a coarse powder. Equal quantity of powder was passed through 40 mesh

sieve and extracted with ethanol (90% v/v) in soxhlet apparatus at 60°C (Chattopadhyay 2003). The solvent was completely removed by rotary vacuum evaporator. The extract was freeze dried and stored in a vacuum desiccator.

Animals: Studies were carried out using Wistar albino male rats (150-200g), obtained from Indian Veterinary Preventive medicine (IVPM), Ranipet, Tamilnadu, India. The animals were grouped and housed in polyacrylic cages (38 x 23 x10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature $25 \pm 2^\circ\text{C}$) with dark and light cycle (12/12 h). The animals were fed with standard pellet diet supplied by Poultry Research Station, Nandhanam, India and fresh water *ad libitum*. All the animals were acclimatized to laboratory condition for a week before commencement of experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Acetaminophen induced nephrotoxicity in rats: Animals were randomized and divided into four groups (I – IV) of six animals in each group. Group I served as untreated control and was fed orally with normal saline 5 ml/kg body weight daily for 7days. Group II rats were similarly treated as group I. Groups III and IV animals were treated with 250 mg/kg and 500 mg/kg body weight of the ethanol extract of *Acorus calamus* for 7 days, respectively. On the 7th day, acetaminophen suspension was given by oral route, in a dose of 750 mg/kg body weight to all rats except the rats in group I.

Hematological study: After 48 h, animals were sacrificed by chloroform anaesthesia. Blood samples were collected by cardiac puncher under diethyl ether anesthesia, using 21 gauge (21 G) needles mounted on a 5ml syringe (Hindustan syringes and medical devices ltd, Faridabad, India.) into ethylene diamine tetra-acetic acid (EDTA) – coated sample bottles for analyzed Hematological parameters like full blood count (FBC), hemoglobin, (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet concentration (PLC) and Total leucocyte count (TLC). These parameters were analyzed using automatic hematological system (Sysmex Hematology – Coagulation system, Model MO-1000 I, Trans Asia, Japan).

Sampling and biochemical analysis: Following termination of the experiment on the day 7, the rats were fasted overnight for 14 hours. Blood samples were collected by cardiac puncture with 21G needle mounted on 5 ml syringe (under diethyl ether anesthesia) and centrifuged for 10min at 5000 rpm. The obtained clear sera were stored at -20°C for subsequent measurement of blood urea, creatinine and uric acid levels using colorimetric assay kits, Bayer (Seamon) according to the manufacturer's instructions.

Preparation of renal homogenate: The kidneys were removed and dissected free from the surrounding fat and connective tissue. Each kidney was longitudinally sectioned, and renal cortex was separated and kept at -8°C . Subsequently, renal cortex was homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The renal cortical homogenates were centrifuged at 5000 rpm for 10 min at 4°C . The resulting supernatant was used for the determination of malondialdehyde (MDA) content, reduced glutathione (GSH) levels and antioxidant enzyme levels such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRD) and glutathione peroxidase (GPX) activity using colorimetric assay.

Biochemical estimation of markers of oxidative stress: MDA content was measured according to the earlier method reported (Zhang 1992). SOD activity was determined according to the previous report (Rai et al. 2006) CAT activity was determined from the rate of decomposition of H_2O_2 by the reported method (Bergmeyer et al. 1974). GPx activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3 (Hafemann et al. 1974). Glutathione reductase activity was assayed according to the previous reports (Carlberg and Mannervik 1975,

Mohandas et al. 1984). Protein content in the tissue was determined by the method reported earlier (Lowry et al. 1951) using bovine serum albumin (BSA) as the standard.

Histopathological examination: Pieces of kidney from each group were fixed immediately in 10% neutral formalin for a period of at least 24 h, dehydrated in graded (50–100%) alcohol, embedded in paraffin, cut into 4–5 μm thick sections and stained with hematoxylin–eosin. The sections were evaluated for the pathological symptoms of nephrotoxicity such as necrosis, fatty infiltration, fibrosis, lymphocyte infiltration, etc.

Results

Effect of Acorus calamus extract on serum urea, uric acid and creatinine concentrations: Serum urea and creatinine concentrations were significantly increased ($p < 0.01$) in the APAP treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity (Figures 2 and 4). Treatment with the ethanol extract of *Acorus calamus* showed significant ($p < 0.05$ and $p < 0.01$) (Group III and IV) decrease in concentrations of serum urea and creatinine compared to the APAP treated group. However the levels of uric acid (UA) significantly decreased ($p < 0.01$) in the APAP treated groups (Group II, Figure 3), when compared to the control group. Treatment with ethanol extract of *Acorus calamus* significantly ($p < 0.05$ and $p < 0.01$) (Group III and IV respectively) increased the uric acid levels, compared to the APAP treated group.

Effect of ethanol extract of Acorus calamus on hematological parameters: APAP caused a significant ($P < 0.01$) increase in the levels of Hb, PCV, DLC and MCV (Figures 2 and 4) (Group II) when compared to the normal control group (Group I), resulting in acetaminophen associated nephropathy. Administration of ethanol extract of *Acorus calamus* significantly (Group III and Group IV; $p < 0.05$, $p < 0.01$ respectively) decreased the Hb, PCV, DLC and MCV levels as compared to the APAP induced group (Group II) (Figure 2 and 4). Further, in APAP treated group (Group II), the levels of PLC, MCHC, MCH and lymphocyte are decreased significantly ($p < 0.01$) when compared with normal (Group I) (Figures 1, 2 and 3). Administration of *Acorus calamus* ethanol extract ensures that these levels are retrieved normally, significantly ($P < 0.05$, $P < 0.01$) when compared with Group 2.

Effect of the Acorus calamus extract on kidney antioxidant status: The activity of CAT in the APAP treated group was significantly ($p < 0.01$) decreased when compared to the normal animals (Group I). Treatment with the ethanol extract of *Acorus calamus* significantly ($p < 0.05$ and $p < 0.01$) (Group III and IV) prevented decrease in the level of catalase activity (Figure 1) compared to the APAP induced rat (Group II). Like wise, the decreased GPx activity as a result of the treatment with APAP was also restored by the *Acorus calamus* extract ($p < 0.05$ and $p < 0.01$) (Figure 2) for Group III and IV as compared to the normal group. Renal SOD activity was decreased significantly ($p < 0.01$) in the APAP treated (group II) animals compared to normal group. Treatment with the ethanol extract of *Acorus calamus* (250 and 500 mg/kg body wt) (Group III and IV) significantly ($p < 0.05$ and $p < 0.01$ respectively) elevated the SOD levels as compared to the APAP induced (Group II) animals (Figure 5). The GSH and MDA levels of APAP and extract treated animals are presented in (Figures 2 and 3). The GSH level reduced significantly ($p < 0.01$) along with increased in MDA concentration in the APAP treated group as compared to the Group I. However on treatment with *Acorus calamus* ethanol extract, the

GSH level was found to be enhanced significantly ($p < 0.05$ and $p < 0.01$) and the MDA contents were reduced in Group III and IV as compared to the induced group (Group II) (Figure 2).

Histopathological studies: The biochemical results were also confirmed by the histological pattern of normal kidney showing normal tubular brush borders and intact glomeruli and Bowman's capsule (Figure 6 (A)). Treatments with acetaminophen sever tubular necrosis and degeneration has shown in the renal tissue (Figure 6 (B)). The rats treated with ethanolic extract of *Acorus calamus* (250mg/kg body weight) showed normal tubular pattern with a mild degree of swelling, necrosis and degranulation (Figure 6(C)) treatment with the extract (500 mg/kg body weight) ameliorated the toxic manifestations in the kidney (Figure 6 (D)).

Figure 1. Effect of treatment with ethanol extract *Acorus calamus* on the renal intracellular CAT activity and blood haematological parameters (Neutrophil, MCHC & MCH), in rats with acetaminophen (APAP)-induced nephrotoxicity. All values are mean \pm S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett's t-test).

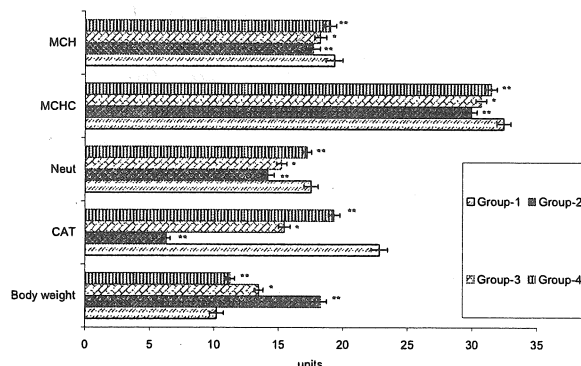


Figure 2. Effect of treatment with ethanol extract of *Acorus calamus* on the renal intracellular GPX, GSH activity, blood Hematological parameters (Gran, TLC and Hb) and serum urea (UR) levels, in rats with acetaminophen (APAP)-induced nephrotoxicity. All values are mean \pm S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett's t-test).

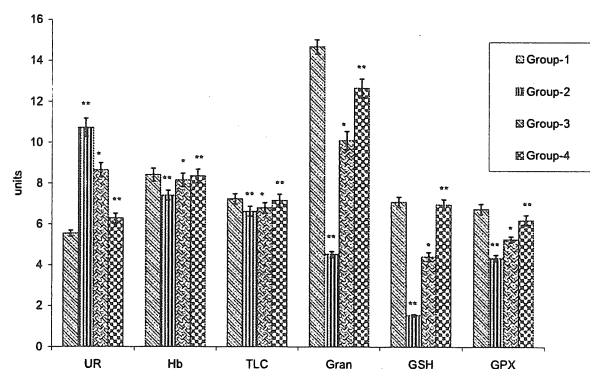


Figure 3. Effect of treatment with ethanol extract of *Acorus calamus* on the renal MDA level, blood hematological parameter (PLC) and serum uric acid levels, in rats with acetaminophen (APAP)-induced nephrotoxicity. All values are mean \pm S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control (One way ANOVA followed by Dunnett's t-test).

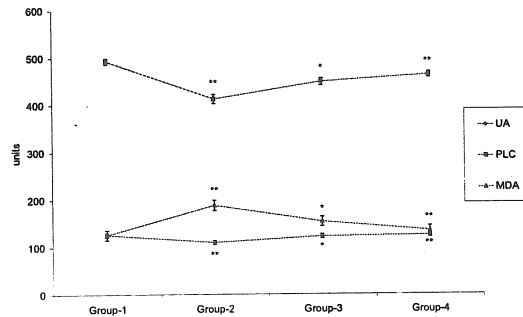


Figure 4. Effect of treatment with ethanol extract of *Acorus calamus* on the blood hematological parameter (MCV, DLC, PCV) and serum creatinine levels, in rats with acetaminophen (APAP)-induced nephrotoxicity. All values are mean \pm S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett's t-test).

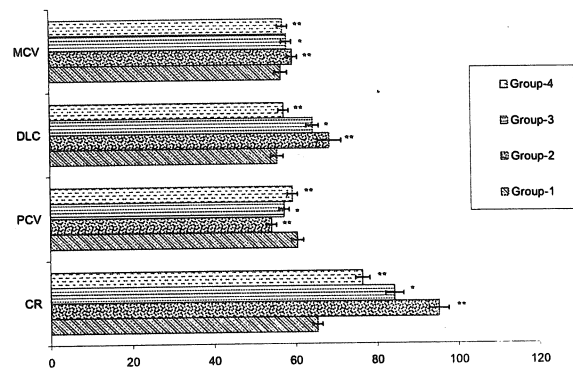


Figure 5. Effect of treatment with ethanol extract of *Acorus calamus* on renal SOD activity in rats with acetaminophen (APAP)-induced nephrotoxicity. All values are mean \pm S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett's t-test).

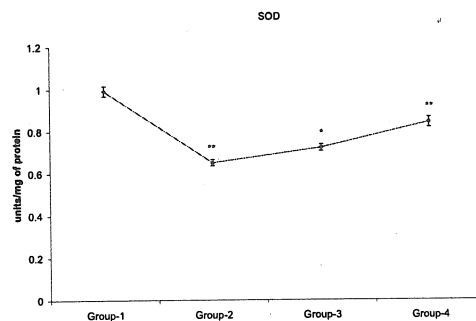
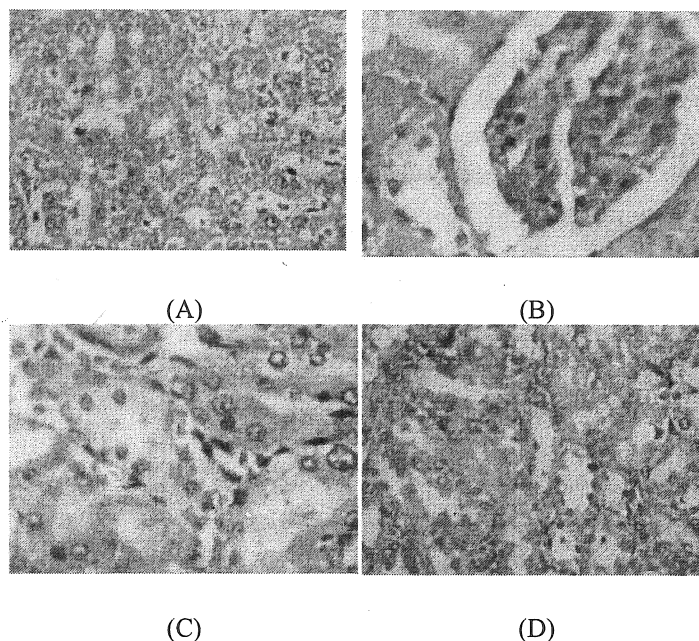


Figure 6. Nephroprotective effect of *Acorus calamus*. Histopathological observations (kidney sections stained with Hematoxylin-Eosin, magnification-100x) (A) Normal, (B) Acetaminophen(APAP), (C) Extract 250mg/kg + APAP, (D) Extract 500 mg/kg + APAP.



Discussion

Acetaminophen over dose is often linked to many metabolic disorders including serum electrolyte, urea and creatinine dearrangements. Increased concentration of serum urea and creatinine are considered for investigating drug induced nephrotoxicity in animals and man. (Bennit et al. 1982). The vital function that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication by xenobiotics, makes the hematopoietic system unique as a target organ (Adeneye et al. 2008). The various blood cells (erythrocytes, leucocytes, and platelets) are produced at a turnover rate of about 1 to 3 million per second in a healthy human adult and this value could be altered in certain physiological or pathological states including hemolytic anemia or suppressive inflammation (Guyton 1991). Certain drugs including alkylating cytotoxic agents could also affect blood formation rate and the normal range of hematological parameters (Adeneye et al. 2008). In the present study, treatment with APAP oral dose significantly increased the Hb, PCV, DLC and MCV levels. After administration of *Acorus calamus ethanol* extract, these levels are significantly decreased compare to the APAP induced group. Whereas the levels of granulocyte, MCH, MCHC and PLC were decreased significantly in the APAP treated group, compared to the normal control group. However after administration of *ethanol* extract of *Acorus calamus* these levels are significantly increased compared to the APAP treated group. However this study shows that the *Acorus calamus* extract could contain candidate molecules reversing the hematotoxic effect of acetaminophen, with ensuing improvement of hematopoiesis. Blood urea nitrogen is found in the liver protein that is derived from diet or tissue sources and is normally excreted in the urine. In renal disease, the serum urea accumulates because the rate of serum urea production exceeds the rate of clearance (Mayne 1994). Elevation of urea and creatinine levels in the serum was

taken as the index of nephrotoxicity (Anwar et al. 1999, Bennit et al. 1982, Ali et al. 2001). Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatinine breakdown (Anwar et al. 1999). Thus serum urea concentration is often considered a more reliable renal function predictor than serum creatinine. In the present study, administration of hepatotoxic and nephrotoxic doses of APAP to rats resulted in development of oxidative stress damage in hepatic and renal tissues. In this study, APAP induced nephrotoxicity showed a significant ($P < 0.01$) increase in the serum urea and creatinine concentrations in the Group II (APAP induced) rat when compared to the normal group (Group I). Moreover, oral administration of ethanolic extract of *Acorus calamus* significantly ($P < 0.01$) decreased in group III and IV when compared to the Group II. However the level of uric acid is significantly decreased ($P < 0.01$) in the Group II rats when compared to Group I. Oral administration of plant extract significantly ($P < 0.01$) increases the uric acid level in Group I when compared to the APAP induced rats (Group II). Thus, oxidative stress and lipid peroxidation are early events related to radicals generated during the hepatic metabolism of APAP. Also the generation of reactive oxygen species has been proposed as a mechanism by which many chemicals can induce nephrotoxicity (Somani et al. 2000). Previous studies have clearly demonstrated that acute APAP overdose increases the lipid peroxidation and suppresses the antioxidant defense mechanisms in renal tissue (Abdel Zaher et al. 2007, Ghosh and Sil 2007). However in the APAP treated animals the MDA levels are increased significantly, when compared to normal control rats. On administration of ethanol extract of *Acorus calamus*, the levels of MDA decreased significantly when compared to APAP induced rats. During kidney injury, superoxide radicals are generated at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages kidney. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism (Pande et al. 2002, Linares et al. 2006). The present study also demonstrated that acute APAP overdose resulted in a decrease in the SOD, CAT and GST activities, when compared with normal control rats. It is due to enhanced lipid peroxidation or inactivation of the antioxidative enzymes. When rat was treated with the ethanol extract of *Acorus calamus*, the reduction of SOD, CAT and GST activity was increased significantly when compared with induced group ($P < 0.01$) (Group II). Current evidence suggests that intracellular GSH plays an essential role in detoxification of APAP and prevention of APAP-induced toxicity in the liver and kidney (Newton et al. 1996, Richie et al. 1992, Nelson 1990). However, APAP was found to increase the microsomal superoxide and hydrogen peroxide production in mice. The generation of the reactive oxygen species appears as an early event which precedes intracellular GSH depletion and cell damage in APAP hepatotoxicity (Manov et al. 2003). APAP administration also caused a significant decrease in GSH content. Administration of ethanol extract of *Acorus calamus*, helped to uplift the GSH depletion induced by APAP. APAP-induced nephrotoxicity was evidenced by biochemical measurements and histopathological changes that coincide with the observations of other investigators (Corcoran et al. 1985, Gardner et al. 2002, Newton et al. 1983). The biochemical results were also confirmed by the histological findings which showed preservation of the glomeruli and the surrounding Bowman's capsule and mildly swollen tubules. Other nephroprotective medicinal plants have been reported of inhibiting xenobiotic-induced nephrotoxicity in experimental animal models

due to their potent anti-oxidant or free radicals scavenging effects (Devipriya et al. 1999, Annie et al. 2005). In addition, alkaloids have also been reported to strongly inhibit lipid peroxidation induced in isolated tissues via its antioxidant activity (Kumaran and Karunnakaran 2007). The protection offered by the extract could have been due to the presence of alkaloids (Talapatra et al. 1968). The activity elicited by the extract might be due to its ability to activate antioxidant enzymes. The findings suggest the potential use of the ethanolic extract of *Acorus calamus* as a novel therapeutically useful nephroprotective agent. Therefore, further studies to elucidate their mechanisms of action should be conducted to aid the discovery of new therapeutic agents for the treatment of renal diseases.

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