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Full Length Research Paper

Toxicological evaluation of precocene II isolated from *Ageratum conyzoides* L. (Asteraceae) in Sprague Dawley rats

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Precocene II (6,7-dimethoxy-2,2-dimethyl-2-chromene) was the main constituent isolated from *Ageratum conyzoides L.* and reportedly possessed antifungal activity. The study investigated the isolation, purification and toxicological effects of precocene II from *A. conyzoides* in Sprague Dawley rats. Precocene II was isolated from the petroleum ether fraction of the plant and the structure was determined by ^{1}H-,^{13}C-,DEPT-NMR and MS spectral techniques. Three groups of eight rats per group were used for the study. While groups B and C were respectively administered with 25 and 50 mg/kg of precocene II in 0.25% CMC-Na for 11 days by gastric intubation, group A was administered with 0.25% CMC-Na and served as the control group. After the last treatment, animals were fasted overnight and on the 12th day, they were injected intravenously with 0.2 ml/kg body weight of phenobarbital. Animals were subsequently dissected from the abdominal region; blood was collected from the pulmonary vein into EDTA anti-coagulated and non anti-coagulated tubes. The liver, kidney and spleen tissues were extracted into separate bottles for histopathological examinations. Results from hematological study indicated that the white blood cell (WBC), red blood cell (RBC), plateletcrit (PCT) and mean corpuscular hemoglobin count (MCHC) were significantly higher across the treated groups. Biochemical result showed that serum glucose level was significantly reduced in the treated groups. No apparent damage was noticed in the liver, kidney and spleen tissues. The result therefore suggests that precocene II possesses hypoglycemic property and could alter some hematopoietic elements but was not toxic to the liver, kidney and spleen tissues.

Key words: *Ageratum conyzoides*, precocene II, isolation, toxicological studies, hematological markers, hypoglycemic property.

INTRODUCTION

Ageratum conyzoides L. belongs to the family of asteraceae and native to Central America, Caribbean, Florida (USA), Southeast Asia, South China, India, West African (including Nigeria), Australia and South America (Okunade, 2002; Kong, 2006). *A. conyzoides* has been widely reported to contain many bioactive compounds which are responsible for its diverse biological activities. The phytochemical compounds mostly isolated were from the oil extract of the plant in which precocene I and II are the major constituents (Okunade, 2002). Precocene I (7-methoxy-2,2-dimethyl-2-chromene) and precocene II (6,7-dimethoxy-2,2-dimethyl-2-chromene) have been used as insect regulators by inducing symptoms of juvenile deficiency hormone in insects (Schrankel et al., 1982). Metabolism of precocenes in insects and rats has been previously studied (Hsia et al., 1981; Haunerland

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and Bowers, 1985) and parallel views have been drawn between the formation of carcinogenic bay region diol epoxides from polycyclic aromatic hydrocarbons in mammals and the bioactivation of precocenes by insects and rats (Pratt et al., 1980; Halpin et al., 1982). Various species of *"Streptomyces*," "*Aspergillus*," "*Rhodotorula*," "*Brevilegnia*," "*Syncephalastrum*" and "*Stysanus*" were found to transform precocene II to three major metabolites identified as cis- and (+) trans-precocene II-3,4-dihydrodiols and (+)-3-chromenol. $^{18}0_2$ incorporation studies indicated the involvement of a monooxygenase enzyme system in precocene II transformation by *Streptomyces griseus* (Sariaslani et al., 1987). Precocene II has also been reported to completely inhibit two species of fungi, *Rhizoctonia solani* and *Sclerotium rolfsii* (Iqbal et al., 2004). Precocene II was found to be a more potent analog and selectively destroys the corpora allata of insects, terminating production of the juvenile hormone. Precocenes are now widely used as tools in experimental arthropod endocrinology and have been considered prototypes of "fourth-generation" pesticides (Sariaslani et al., 1987). Toxicity studies of naturally derived precocene are lacking and to the best of the present knowledge, there has been no detailed report of hematological parameters on animals treated with precocene II. Thus, this research investigated the toxicological effects of naturally occurring precocene II isolated from *A. conyzoides* in Sprague Dawley (SD) rat using biochemical, hematological and histopathological indices of toxicity.

MATERIALS AND METHODS

Plant collection and identification

The whole plant of *A. conyzoides* L. was obtained in April, 2008 from Xushuangbanna, South Western China and authenticated by Dr. You-Kai Xu of the Xushuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, China. A voucher specimen (KUN 0486260) was subsequently deposited in the Herbarium of Kunming Institute of Botany-CAS, China.

Plant extraction, isolation and structure elucidation

The dried whole plant (7 kg) of *A. conyzoides* was extracted with 95% ethanol for three times (each for one week) at room temperature. After concentration of the combined extracts under reduced pressure, the residue was dissolved in hot water and extracted successively with petroleum ether, ethyl acetate and n-butanol. The petroleum ether (201 g) was fractionated by column chromatography (CC) (268 g silica gel; Pet. Ether /Acetone 1:6, 1:3 and 1:1) to afford several fractions. The first fraction was purified by repeated CC (1. sephadex LH-20, $CHCl_3$: MeOH 2:1 and 2. SiO_2; Pet. Ether./$CHCl_3$ 1:2, 10:1, to afford precocene II (2.1 g). Precocene II was obtained as yellow oil. Its molecular weight from EI-MS (VG Autospec-3000, UK) was 220. From the ^{1}H, ^{13}C-NMR and DEPT spectra (Bruker AM-400 and DRX-500, Rheinstteten, Germany), the molecular formular, $C_{13}H_{16}O_3$ was derived. Furthermore, the ^{1}H and ^{13}C-NMR data showed that this compound was a chromene and when compared with literatures, it was identified as 6, 7-dimethoxy-2,2-dimethyl-2-chromene (precocene II) (Gonzalez et al., 1991; Iqbal et al., 2004). (6,7-dimethoxy-2,2-dimethyl-2-chromene, Precocene II), pale yellow oil, $C_{13}H_{16}O_3$, ^{1}H-NMR (500 MHz, CD_3OD) δ_H: 5.47(1H, d, J = 9.7 Hz, H-3), 6.24(1H, d, J = 9.7, H-4), 6.53(1H, s, H-5), 6.41(1H, s, H-8), 1.41(6H, s, 2x CH_3 at C-2), 3.77(6H, s, 2x –OCH_3 at C-6,7). ^{13}C-NMR (125 MHz, CD_3OD), δc: 75.9(s, C-2), 128.2(d, C-3), 121.9(d, C-4), 149.6(s, C-4a), 109.7(d, C-5), 143.0(s, C-6), 147.2(s, C-7), 101.0(d, C-8), 113.0(s, C-8a), 27.6(q, 2x CH_3, C-9/10), 56.5(q, –OCH_3, C-13), 55.8(q, -OCH_3, C-14). EI-MS *m/z* (%): 220 $[M]^+$ (30), 205 (34), 191 (25), 177 (36), 165 (95), 95 (100), 81 (62), 69 (44) (Figure 1).

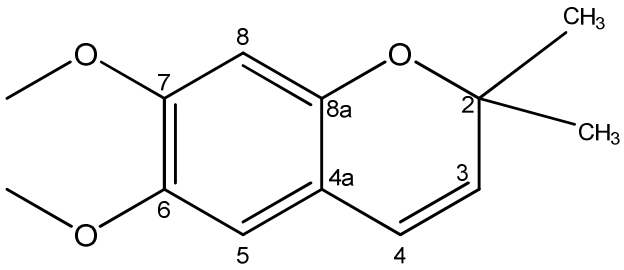

Figure 1. Structure of precocene II.

Experimental animals

Twenty-four male SD rats, specific pathogen free (SPF), aged 7 - 9 weeks and weighing 220 – 265 g were purchased from Vital River Laboratories, Beijing, China and transported by air to the Animal House of the Institute of Toxicology, Kunming Medical University, Kunming, China. They were kept under standard environmental conditions (25 $\pm$ 2℃; 12/12 h light/dark cycle). Four animals were housed in each cage and fed with standard diet (obtained from Yunnan Key Laboratory of Animal Nutrition and Feed Stuff, Yunnan Agriculture University, Kunming, China) and water *ad libitum*. The animals were allowed to acclimatize before beginning the experiment. For experimentation, the animals were fasted overnight and 8 animals were included in each group. All animals were treated in compliance with the guidelines of National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication, 1985). Also, the animal protocols of the Institute of Toxicology, Kunming Medical University Ethics Committee on Research in Animals were followed.

Experimental design

In the experimental design, sub-chronic toxicity study was carried out on the compound (precocene II) isolated from *A. conyzoides*. Two groups (B and C) of eight rats per group were respectively administered 25 and 50 mg/kg body weight doses of precocene II in 0.25% CMC-Na for 11 days via gastric intubation. Animals in group A (control group) also made up of eight rats were administered with 0.25% CMC-Na per kg body weight. On the 12th day, animals were injected intravenously with 0.2 ml/kg body weight of phenobarbital (sedative). They were subsequently dissected from the abdominal region; blood was collected from the pulmonary vein using the vacutainer collection set into EDTA anti-coagulated and non anti-coagulated bottles. The liver, kidney, heart, spleen, brain, lungs, thymus, testes, epidydimis and adrenal gland were extracted into separate bottles for histopathological examinations. The blood sample collected into EDTA bottles was used for hematological studies while the blood in non anti-coagulated bottles was centrifuged at 1,500 g for 10 min, and the sera collected into clean, dried tubes for biochemical analyses.

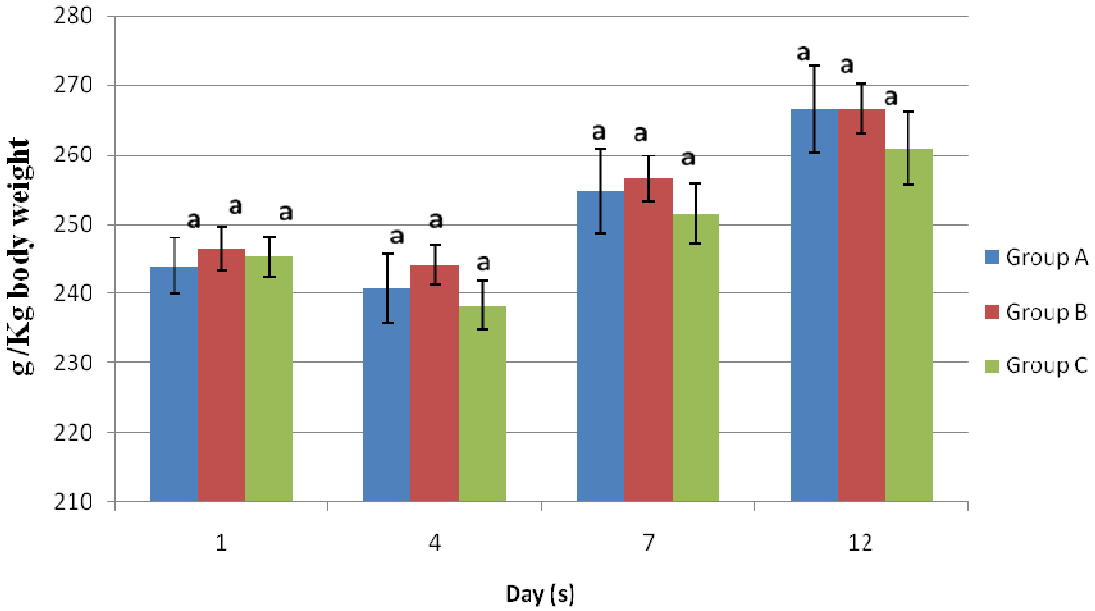

Figure 2. Weights of Sprague Dawley rats treated with precocene II isolated from *Ageratum conyzoides*. Values are represented as mean ± SEM of 8 replicates. Values on the same day with different letters differ significantly ($p < 0.05$).

Biochemical and hematological assays

Commercial test kits obtained from Biosino Bio-Technology and Science Inc. Beijing, China were used for all biochemical parameters measured. Standard methods were used to estimate glucose (Trinder, 1969), aspartate aminotransferase (AST) (Bergmeyer et al., 1986a), alanine aminotransferase (ALT) (Bergmeyer et al., 1986b), alkaline phosphatase (ALP) (Tietz et al., 1983), creatine kinase (CK) (Chemnitz et al., 1979), total protein (Weichselbaum, 1946), albumin (Doumas et al., 1971), total bilirubin (Doumas et al., 1973), urea (Krieg et al., 1986), creatinine (Larsen, 1971), triglycerides and cholesterol (Zoppi and Fellini, 1976). These parameters were determined using the automated biochemical muti-item analyzer (TMS-1024; Tokyo Boeki Medical System Ltd., Japan). The whole blood was used to assay white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), percentage lymphocyte, percentage monocyte, percentage granulocyte, platelet distribution width (PDW) and plateletcrit (PCT) using the techniques described by Baker et al. (1998). These parameters were determined by the automated hematology system analyzer (ADVIA 60 Open Tube; Bayer Corporation Tarrytown New York, USA).

Histopathological studies

The method described by Aliyu et al. (2007) with slight modification was followed. After blood collection, the liver, kidney and spleen were carefully dissected from the abdominal region. They were fixed in normal saline for 72 h and sliced into a thickness of 2.1 mm. The tissues were dehydrated with alcohol of graded concentrations. They were further treated with paraffin wax and cast into blocks; sections of the tissues were then cut on a microtome to 5 µm. These were later attached to a slide and allowed to dry. The sample slides were subsequently stained in haematoxylin-eosin and examined under a light microscope; photomicrographs of the samples were recorded.

Statistical analysis

The differences among experimental and control groups were determined using Statistical Package for the Social Sciences (SPSS) for Window XP software programme (version 13.0). Group comparisons were done using the analysis of variance (ANOVA) test. Significant difference between control and experimental groups were assessed by least significant difference (LSD) and the student's *t*-test. All data were expressed as mean ± standard error of mean (SEM); p-values less than 0.05 were considered to be significant.

RESULTS

Animal weights and weight of organs

No significant difference ($p > 0.05$) was observed in the weight of animals and organs of rats treated with precocene II as compared with the control (Figure 2 and Table 3).

Hematological and biochemical assays

The WBC was significantly higher ($p < 0.01$) in the group treated with 50 mg/kg body weight; similarly, the RBC was significantly elevated ($p < 0.05$) in the same group when compared with the control group. There were

Table 1. Effects of precocene II on biochemical parameters of Sprague Dawley rats.

Parameters	Group A	Group B (mg/kg body weight)	Group C
	Control	25	50
Glucose (mmol/L)	7.01 ± 0.31[a]	6.04 ± 0.46[b]	5.70 ± 0.11[c]
ALT (U/L)	42.87 ± 2.22 [a]	38.72 ± 3.38[a]	47.03 ± 5.18[a]
AST (U/L)	98.92 ± 3.21[a]	103.54 ± 4.79[a]	105.56 ± 5.07[a]
ALP (U/L)	226.97 ± 8.17[a]	233.53 ± 12.21[a]	217.96 ± 15.73[a]
Total Bilirubin (µmol/L)	2.28 ± 0.22[a]	2.43 ± 0.10[a]	2.61 ± 0.07[a]
Total Protein (g/L)	55.39 ± 0.90[a]	57.07 ± 1.02[a]	56.07 ± 0.07[a]
Albumin (g/L)	36.34 ± 0.40[a]	36.86 ± 0.47[a]	36.38 ± 0.34[a]
Albumin/Globulin	1.95 ± 0.10[a]	1.84 ± 0.05[a]	1.86 ± 0.04[a]
Creatine kinase (U/L)	858.19 ± 151.70[a]	1079.39 ± 125.69[a]	656.94 ± 132.28[a]
Urea (mmol/L)	6.60 ± 0.34[a]	6.35 ± 0.16[a]	7.21 ± 0.43[a]
Creatinine (µmol/L)	25.14 ± 1.31[a]	26.15 ± 0.75[a]	27.39 ± 0.78[a]
Total Cholesterol (mmol/L)	1.51 ±0.09[a]	1.45 ± 0.07[a]	1.59 ± 0.08[a]
Triglyceride (mmol/L)	0.46 ± 0.03[a]	0.50 ± 0.04[a]	0.36 ± 0.07[a]

Values are represented as mean ± SEM of 8 replicates. Values on the same row followed by different superscript letters differ significantly ([b]$p < 0.05$; [c] $p < 0.01$ vs control).

Table 2. Effects of precocene II on the hematological parameters of Sprague Dawley rats.

Parameters	Group A	Group B (mg/kg body weight)	Group C
	Control	25	50
WBC (x 10^{12}/L)	4.88 ± 0.32[a]	5.54 ± 0.32[a]	6.25 ± 0.40[c]
RBC (x 10^{12}/L)	6.54 ± 0.16[a]	6.54 ± 0.09[a]	7.22 ± 0.33[b]
HB (g/L)	139.00 ± 3.30[a]	144.29 ± 2.37[a]	150.50 ± 7.02[a]
HCT (L/L)	0.33 ± 0.02[a]	0.33 ± 0.01[a]	0.36 ± 0.02[a]
PLT (x 10^{9}/L)	1097.25 ± 44.99 [a]	1333.14 ± 98.78 [a]	1253.75 ± 82.31[a]
PCT (x 10^{-2}L/L)	0.75 ± 0.38[a]	0.98 ± 0.11[b]	0.97 ± 0.08[b]
MCV (fL)	50.75 ± 0.86 [a]	50.86 ± 0.59[a]	50.38 ± 0.82[a]
MCH (pg)	21.28 ± 0.43[a]	22.07 ± 0.25[a]	20.86 ± 0.37[a]
MCHC (g/L)	419.00 ± 3.86[a]	434.57 ± 6.07[b]	415.25 ± 5.15[a]
RDW (%)	15.11 ± 0.48[a]	15.03 ± 0.52 [a]	15.14 ± 0.50[a]
MPV (fL)	6.78 ± 0.14 [a]	7.34 ± 0.29 [a]	7.25 ± 0.32[a]
PDW (%)	7.13 ± 0.24[a]	7.50 ± 0.31[a]	7.23 ± 0.34[a]
Lymphocyte (%)	90.50 ± 1.78[a]	91.04 ± 2.04[a]	88.51 ± 1.83[a]
Monocyte (%)	7.76 ± 1.32[a]	7.76 ± 1.79[a]	9.46 ± 1.62[a]
Granulocyte (%)	1.74 ± 0.50[a]	1.20 ± 0.38[a]	1.78 ± 0.38[a]

Values are represented as mean ± SEM of 8 replicates. Values on the same row followed by different superscript letters differ significantly ([b]$p < 0.05$; [c] $p < 0.01$). WBC, White blood cell count; RBC, red blood cell count; HB, Hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit.

significant increases ($p < 0.05$) in PCT in both treated groups. MCHC was also statistically higher ($p < 0.05$) in the group treated with 25 mg/kg of precocene II when compared with the control group (Table 2). ALT, AST, ALP, total bilirubin, total protein, albumin, cholesterol, triglycerides, creatine kinase, urea and creatinine were not significantly different ($p > 0.05$) from the control group. Serum glucose concentration was significantly decreased in groups B ($p < 0.05$) and C ($p < 0.01$) (Table 1).

Table 3. Effects of precocene II isolated from *A. conyzoides* on the weight of organs in Sprague Dawley rats.

Organs	Group A	Group B (mg/kg body weight)	Group C
	Control	25	50
Thymus (x 10^{-2}g)	36.09 ± 2.08[a] [13.55 ± 0.76][a]	34.86 ± 1.89[a] [13.07 ± 0.70][a]	36.18 ± 2.37[a] [13.86 ± 0.84][a]
Adrenal gland (x 10^{-2} g)	5.58 ± 0.34[a] [2.09 ± 0.10][a]	5.66 ± 0.32[a] [2.12 ± 0.29][a]	5.65 ± 0.39[a] [2.17 ± 0.15][a]
Heart (g)	0.96 ± 0.03[a] [0.36 ± 0.01][a]	0.91 ± 0.03[a] [0.34 ± 0.01][a]	0.91 ± 0.02[a] [0.35 ± 0.01][a]
Liver (g)	7.36 ± 0.24[a] [2.77 ± 0.09][a]	7.60 ± 0.23[a] [2.85 ± 0.08][a]	7.45 ± 0.27[a] [2.85 ± 0.07][a]
Spleen (g)	0.61 ± 0.06[a] [0.22 ± 0.02][a]	0.54 ± 0.02[a] [0.20 ± 0.01][a]	0.54 ± 0.04[a] [0.21 ± 0.01][a]
Lungs (g)	1.11 ± 0.02[a] [0.42 ± 0.02][a]	1.14 ± 0.03[a] [0.43 ± 0.01][a]	1.15 ± 0.06[a] [0.44 ± 0.02][a]
Kidney (g)	2.04 ± 0.11[a] [0.77 ± 0.05][a]	2.02 ± 0.08[a] [0.76 ± 0.03][a]	1.89 ± 0.06[a] [0.72 ± 0.02][a]
Brain (g)	1.85 ± 0.02[a] [0.70 ± 0.02][a]	1.78 ± 0.02[a] [0.67 ± 0.01][a]	1.81± 0.04[a] [0.70 ± 0.02][a]
Testes (g)	2.87 ± 0.10[a] [1.08 ± 0.05][a]	2.77 ± 0.06[a] [1.04 ± 0.02][a]	2.67 ± 0.05[a] [1.02 ± 0.02][a]
Epididymis (g)	0.81 ± 0.04[a] [0.30 ± 0.02][a]	0.98 ± 0.13[a] [0.37 ± 0.05][a]	0.84 ± 0.06[a] [0.32 ± 0.03][a]

Values are represented as mean ± SEM of 8 replicates; values in parenthesis are % weight of organ with respect to the body weight of rats. Values on the same row followed by different superscript letters differ significantly ($p < 0.05$).

Histopathological studies

No significant damage was observed in rats' liver, kidney and spleen tissues in both groups treated with precocene II isolated from *A. conyzoides* when compared with the control.

DISCUSSION

No deaths occurred during the period of treatment. No changes in locomotor activity or occurrences of piloerection or diarrhea were observed. Changes in body weight are a valuable indicator in evaluating the toxicity of a compound or extract preparation (Grance et al., 2008). The slight drop in weight after 4 days of treatment in all the treated groups could be a normal physiological and adaptational responses mediated by the compound, thus decreasing the appetite and thereby lowering caloric intake by the animals (Rhiouani et al., 2008). The weights subsequently picked up and may suggest that the compound do not exert adverse effects on the animals. From hematological studies, the sub-acute exposure of rats to both lower and higher doses (25 and 50 mg/kg, respectively) of the compound, precocene II produced significant changes in some hematological parameters. The increase in erythrocytes (RBC), mean corpuscular hemoglobin concentration (MCHC), leukocytes (WBC) and plateletcrit (PCT) may be due to overproduction of hematopoietic regulatory elements such as colony-stimulating factors, erythropoietin and thrombopoietin by the stromal cells and macrophages in the bone marrow (Chang-Gue et al., 2003) thus providing the local environment for hematopoiesis (Udut et al., 2005; Rhiouani et al., 2008). From the observed significant increase in the values of WBC, it was clear that an increase in the number of WBC is a normal reaction of rats to foreign substances, which alter their normal physiological processes. The leucocytosis observed in the present study indicates a stimulation of the immune system which protects the rats against infection that might have been caused by chemical and also secondary infections, which may be contracted after the weakening condition of the rats. Leucocytosis, which may be directly proportional to the severity of the causative stress condition, may be attributed to an increase in leukocyte mobilization (Celik and Suzek, 2008). Biochemical markers are often used as basis for the assessment of toxicity of an organ. Thus, in the present study, no significant variations in the levels of AST, ALT, ALP, total protein, albumin, total bilirubin, urea and creatinine was observed thus suggesting the non-toxicity of the

compound to the liver and kidney within the treatment durations. The result from histological sectioning was in agreement as there were no apparent liver and kidney injury observed in all the treated groups when compared to the control groups. This further confirmed that the compound may not be toxic to the liver and kidney within these treatment periods. Diabetes is a chronic metabolic disorder affecting a major population worldwide. A sustained reduction in hyperglycemia will decrease the risk of developing micro vascular diseases and reduce their complications (Kim et al., 2006). It has been established that lowering the plasma glucose level may be induced by the release of insulin, an endogenous peptide involved in the regulation of blood sugar (Cunha et al., 2008). Hence, the measurement of serum glucose level has been used as a marker for diabetes mellitus. Thus, a significant decreased in serum glucose level was observed in rats treated with precocene II thus suggesting that the compound possess hypoglycemic activity. This hypoglycemic effect could be attributed to the potentiation of insulin effect of plasma by increasing the pancreatic secretion of insulin from the existing β-cells or its release from bound insulin (Sharma et al., 2008; Eidi et al., 2006; Kasiviswanath et al., 2005). Previous work has reported a reduction in serum glucose level by 21.3% after 4 h of treatment with the aqueous leaf extract of *A. conyzoides* (Nyunai et al., 2006). Precocene II could be the active phytochemical responsible for the hypoglycemic activity of this plant.

In conclusion, precocene II was found to possess hypoglycemic activity and could alter some hematological elements with no toxicity to the liver, kidney and spleen tissues, and therefore might be used for the treatment of diabetes mellitus. Further investigation is needed to understand the structure-activity relationship and the exact hypoglycemic mechanisms of precocene II.

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