Research Article

Toxicity Evaluation of *A. Bilimbi* L. Fruit Extract on Haematological and Histopathological Analysis in Animal Model

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ABSTRACT

A. bilimbi L., is a common plant in Asia, which belongs to the family of the Oxalidaceae. In Malaysia, this plant is used to remove off-odour of freshwater fish, and is traditionally being used as treatments for cough, cold, viral and bacterial infections, rheumatism, syphilis, diabetes, and hypertension. This study investigated the acute and sub acute toxicity effects of *A. bilimbi* fruit extract, as described in OECD Guidelines 423 and 407, respectively. The LD_{50} value, haematological assessments as well as histopathological analysis were evaluated in both studies. In acute toxicity study, all rats in the tested groups could tolerate the extract up to 5000 mg/kg bwt of dosage. Neither mortality nor treatment related changes in their behaviour and external appearance were observed, indicated that the LD_{50} value of *A. bilimbi* is higher than 5000 mg/kg bwt. In sub acute toxicity study, haematological evaluation showed statistically significant differences in both packed cell volume (PCV) and mean corpuscular haemoglobin concentration (MCHC) values in male treated rats, with 250 mg/kg bwt of dosage. Female treated rats showed significant differences in PCV and MCHC values for 250 mg/kg bwt of dosage, and significant increased in Hb value for treatment of 500 mg/kg, as compared to the control group. Histopathological study revealed normal architectures of all selected organs among *A. bilimbi* treated rats. The finding therefore, revealed that the fruit extract of *A. bilimbi* is not toxic to the animals, with an exception to a minor change in certain haematological parameters.

Keywords: *A. bilimbi*, Acute toxicity, Haematology test, Histopathological analysis, LD_{50}, Subacute toxicity.

INTRODUCTION

Herbal remedies, mainly used in traditional medicine, have been the basis for the treatment of various ailments for many centuries. The exclusive use of herbal remedies for the management of certain diseases continues unabated due to easy access and strong belief that these products are safe.[1] usually; the remedies are administered over a long period of time without a proper dosage monitoring and consideration for its toxic effects. An initial step of assessment and evaluation of the toxic characteristics of a natural product extract, fraction or compound are usually conducted in screening natural products for their pharmacological activity.[2]

A. bilimbi L. (Oxalidaceae), also known as 'Belimbing Buluh' in Malaysia is a tropical plant commonly consumed for its medicinal properties. It is an attractive, long-lived small tree that can grow up to 15 metres high. The fruits are fairly cylindrical with five broad rounded longitudinal lobes, and produced in clusters. Crispy when unripe, the fruit turns from bright green to yellowish-green, ivory or nearly white when ripe and falls to the ground.[3] In Malaysia, these plants are used to wash seafood and especially freshwater fish, in order to remove any of its off-odour. Because of the high oxalic acid content, the fruit juice is useful for bleaching stains on hands, iron-rust from white cloth, and also to impart shine to brassware.[4,5] The high level of organic acid present in this fruit is probably responsible for its very sour taste, where, it is often used in the production of vinegar, wine, pickles and in the preparation of Hindu dishes.[6] Traditionally, it is widely used as treatments for cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough, and hypertension.[7] In both viral and bacterial infections, the fruits of *A. bilimbi* are used in the treatments of acne, mumps, and abscesses[8] and also to treat dyspepsia, colitis and dental caries.[9] It is therefore important to establish scientific information on *A. bilimbi* toxicity effects since the scientific literatures on the safety use of this plant is lacking. Hence, the present research was undertaken to provide first hand information on acute and sub acute toxicity studies of *A. bilimbi* ethanolic extract on animal model.

MATERIALS AND METHODS

Plant Sample Collection and Identification

The fruits of *A. bilimbi* were collected from Universiti Teknologi Mara (UiTM), Selangor, Malaysia. Taxonomical identification of herbarium was done by Faculty of Science and Technology, University Kebangsaan Malaysia, and voucher specimens were deposited there with voucher number UKMB 30018.

Extraction of Test Material

The fresh fruits of *A. bilimbi* were washed; air dried, and ground using cryogenic grinding to form fine powder. Freeze drying method was utilised to remove excess water from the powder. An amount of 1.5 kg of the powder was then soaked in 10.5 L (1:10) of 80% ethanol

for three days at room temperature. The extraction procedure was repeated twice using the same powder. The filtrates from each extraction were mixed and the excess solvent was evaporated under reduced pressure, using a rotary evaporator (Buchi, Rotavapor, Switzerland), to give a dark green crude ethanolic extract.

Experimental Animals

The experiment was performed on healthy male and female Sprague Dawley rats of eight weeks old and body weight of 160-200 g. They were purchased from CheNur Supplier Sdn. Bhd. The female rats were nulliparous and non-pregnant. The rats were fed with standard laboratory diets, given water *ad libitum* and maintained under laboratory conditions of temperature 22°C (± 3°C), with 12 h light and 12 h dark cycle. The experimental procedures involving the handling and treatment of animals were approved by the Universiti Technologi MARA (UiTM) Ethics and Animal Care Committee and the approval number is 16/2011.

Procedure for Acute Oral Toxicity Test

The acute oral toxicity of the crude ethanolic extract of *A. bilimbi* was evaluated in rats using the procedure described by Organization for Economic Co-operation and Development 423 Guidelines.[10] A total of 18 female animals were divided into three dosage groups with 6 animals per dose. The control group was given normal saline at 1ml/100 g of body weight. The second and third groups were given with a single dose of 2000 mg/kg bwt and 5000 mg/kg bwt of *A. bilimbi,* respectively. Gavage dosing was performed using a curved, ball-tipped intubation needle affixed to a 5 ml syringe. Fresh preparation of solutions was made prior to dosing and they were kept chilled and tightly capped.

Initial body weight was recorded prior to dosing. Animals were fasted approximately 12 hours followed by administration of a single dose of *A. bilimbi* extract. The animals were observed for general toxicity signs and behavioural changes. The signs of toxicity included convulsions, tremors, circling, depression, excitement and mortality. Results were recorded for the first 30 minutes and at hourly intervals for the next 24 hours, while food and water consumption were monitored daily for a total of 14 days. Body weight was measured again on Day 7 and Day 14. At the end of the experiment, all of the animals were sacrificed for gross necropsy findings.

Procedure for Sub acute Oral Toxicity Test

Repeated dose oral toxicity study was carried out according to OECD 407 Guideline.[11] The animals were divided into four groups of 12 animals each (6 males and 6 females). Group 1 received normal saline at 1ml/100 g of body weight and served as control. Based on the initial information obtained from acute toxicity studies, three doses were selected for the treatment of sub acute toxicity of the *A. bilimbi* extract. The doses were 125, 250 and 500 mg/kg body weight. Treatments and observations of animals after administration of the extract was carried out for 28 days as reported for the acute toxicity study in the guideline (OECD, 2008). Body weight was measured prior to dosing, weekly and thereafter until necropsy. At the end of experiment the blood was collected via cardiac puncture under mild anaesthesia using diethyl ether. Then, the animals were sacrificed and organs that included heart, liver, lung, kidney, and reproductive organs were collected. They were preserved in 10% formalin to prevent tissue autolysis and further processed for histology. The collected blood was used to evaluate haematological parameters.

Haematological Parameters

Blood samples were collected from anesthetized animals through cardiac puncture into ethylene diamine tetra acetic acid (EDTA) tubes. The blood samples were then analysed for red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb) contents and packed cell volume (PCV) using standard techniques.[12,13]

Histopathological Analysis

Fixed organs were subjected to histological preparation and processing. The paraffin sections of each tissue were cut at 4 µm thickness and stained with haematoxylin and eosin for microscopic examination. Histopathological analysis was conducted for gross pathology and the presence of any lesions.

Statistical Analysis

All haematological parameters for both treated and control rats were analysed using the One-way Analysis of Variance (ANOVA). A p value of 0.05 or less ($p<0.05$) was considered to be significant. All data were expressed as means ± standard error of the means.

RESULTS AND DISCUSSION

Acute Toxicity Study

No mortality and treatment related changes were observed in behaviour and external appearance of rats following single oral administration of both 2000 mg/kg and 5000 mg/kg of *A. bilimbi* extract. This result indicated that the LD_{50} value of *A. bilimbi* is higher than 5000 mg/kg.

Sub acute Toxicity Study

No behavioural changes, toxicity signs and death were observed at the end of the treatment period (28 days) in both rats genders of rats. Table 1 and Table 2 show haematological results for sub acute treatment of *A. bilimbi*. Haematological parameters revealed statistically significant differences in PCV and MCHC with the dose of 250 mg/kg in male treated rats. In female treated rats, there was a significant increased in Hb value for treatment of 500 mg/kg, while PCV and MCHC showed significant differences for dose of 250 mg/kg as compared with control group.

Table 1: Haematological values of sub acute treatment with *A. bilimbi* fruit ethanolic extract of male rats

Treatment Groups	RBC (x10^6)	WBC (x10^3)	PCV (%)	Hb (g/dl)	MCV (µm^3)	MCH (pg)	MCHC (%)
Control	9.44±1.33^a	12.22±2.73^a	42.00±3.38^a	14.03±0.37^a	50.48±10.18^a	17.38±3.85^a	34.64±3.14^b
125 mg/kg	8.76±0.50^a	10.53±2.46^a	36.83±3.11^{ab}	14.03±0.37^a	42.17±2.82^a	16.27±0.97^a	39.07±2.37^{ab}
250 mg/kg	8.64±0.41^a	14.01±3.33^a	31.17±1.33^b	4.03±0.37^a	36.53±2.45^a	16.36±0.69^a	45.33±1.90^a
500 mg/kg	9.85±0.28^a	11.67±2.37^a	39.00±1.65^a	14.85±0.01^a	39.69±1.69^a	15.15±0.44^a	38.45±1.74^{ab}

Each value represents the mean ± SEM (n = 6); Superscripts $^{a, b}$ within a column showed significant difference at $p<0.05$

Table 2: Haematological values of sub acute treatment with *A. bilimbi* fruit ethanolic extract of female rats

Treatment Groups	RBC (x10^6)	WBC (x10^3)	PCV (%)	Hb (g/dl)	MCV (µm^3)	MCH (pg)	MCHC (%)
Control	7.99±0.52^a	7.17±1.80^a	40.17±1.78^a	13.75±0.35^b	51.67±5.05^a	17.72±1.67^a	34.43±1.08^b
125 mg/kg	8.69±0.48^a	9.38±1.61^a	39.00±1.90^{ab}	13.48±0.28^b	45.27±2.28^a	15.80±1.10^a	34.92±1.61^b
250 mg/kg	8.07±0.33^a	8.74±1.98^a	33.33±1.67^b	14.03±0.37^{ab}	41.67±2.77^a	17.49±0.68^a	42.54±2.16^a
500 mg/kg	8.64±0.86^a	10.00±1.49^a	44.17±2.60^a	14.85±0.01^a	53.79±6.92^a	18.20±2.06^a	34.27±2.22^b

Each value represents the mean ± SEM (n = 6); Superscripts $^{a, b, c}$ within a column showed significant difference at $p<0.05$

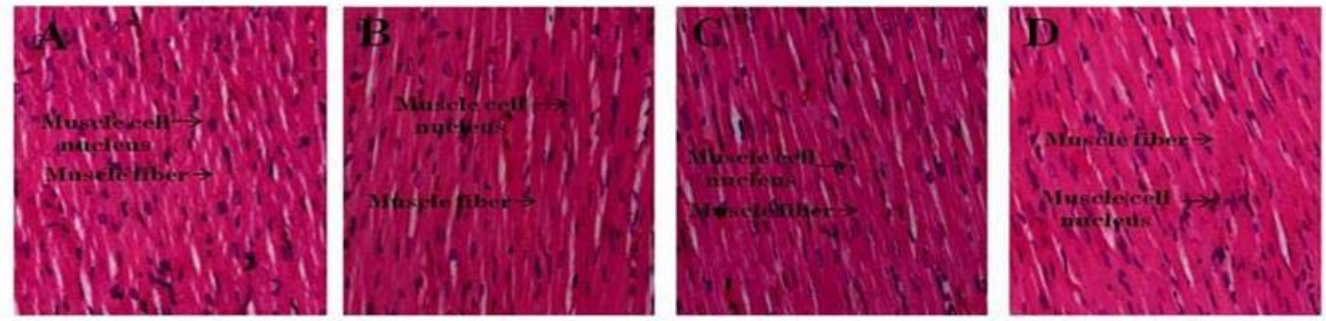

Figure 1: Cross sections of hearts (H & E staining, 20X). (A): Control, (B): 125 mg/kg, (C): 250 mg/kg and (D): 500 mg/kg showed normal histopathological features of cardiac muscles, with the nucleui (arrows) were located centrally.

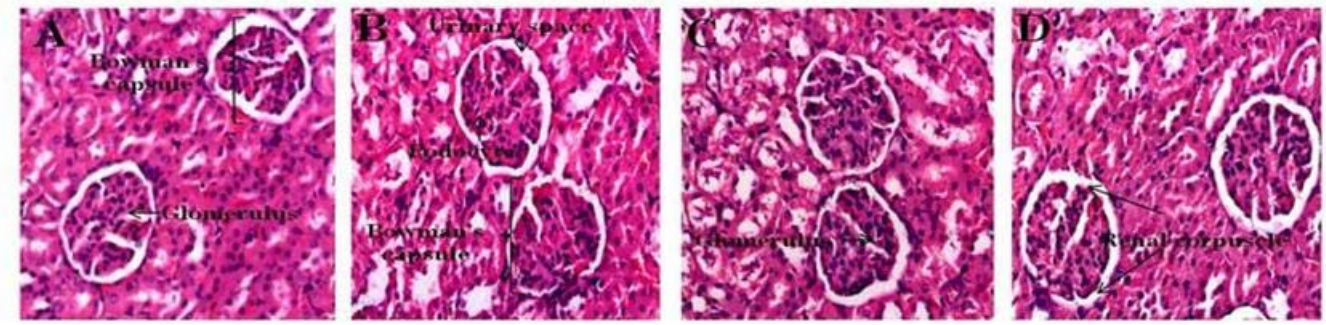

Figure 2: Cross sections of kidneys (H & E staining, 20X). (A): Control, (B): 125 mg/kg, (C): 250 mg/kg and (D): 500 mg/kg showed intact Bowman's capsules (arrows), glomeruli (arrow) and renal tubules.

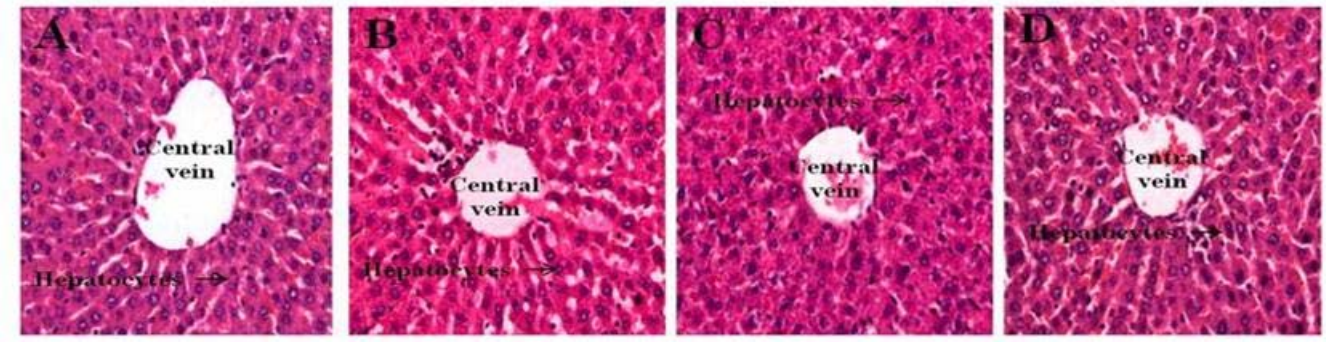

Figure 3: Cross sections of livers (H & E staining, 20X). (A): Control, (B): 125 mg/kg, (C): 250 mg/kg and (D): 500 mg/kg revealed well preserved hepatocytes (arrows) with brought out nuclei and cytoplasm that was also not vacuolated.

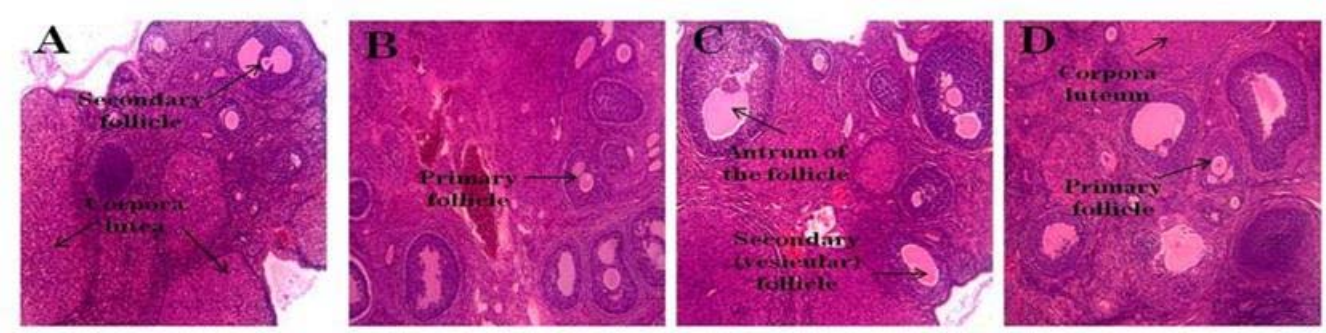

Figure 4: Cross sections of rat's ovaries (H & E staining, 4X). (A): Control, (B): 125 mg/kg, (C): 250 mg/kg and (D): 500 mg/kg animals revealed normal developing follicles and corpora lutea (arrows).

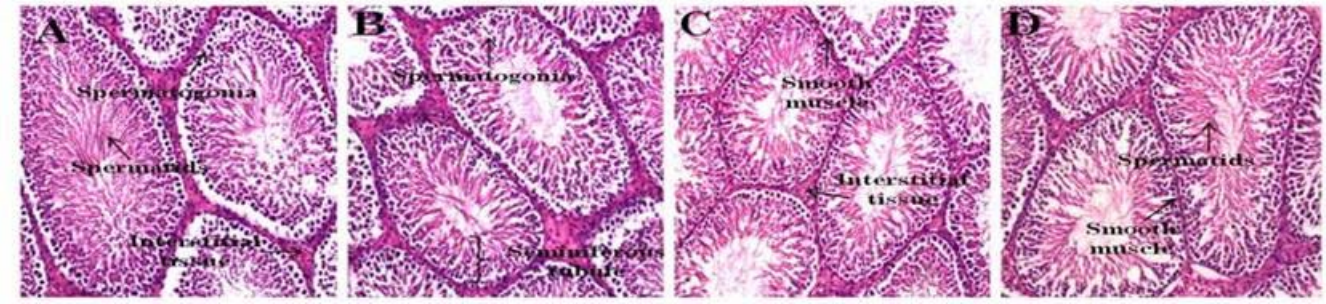

Figure 5: Cross sections of testes (H & E staining, 10X). (A): Control, (B): 125 mg/kg, (C): 250 mg/kg and (D): 500 mg/kg showed normal arrangements of the internal structures of the seminiferous tubules. The tubules consist of various stages of spermatogenic cells and were connected through interstitial space containing interstitial tissues.

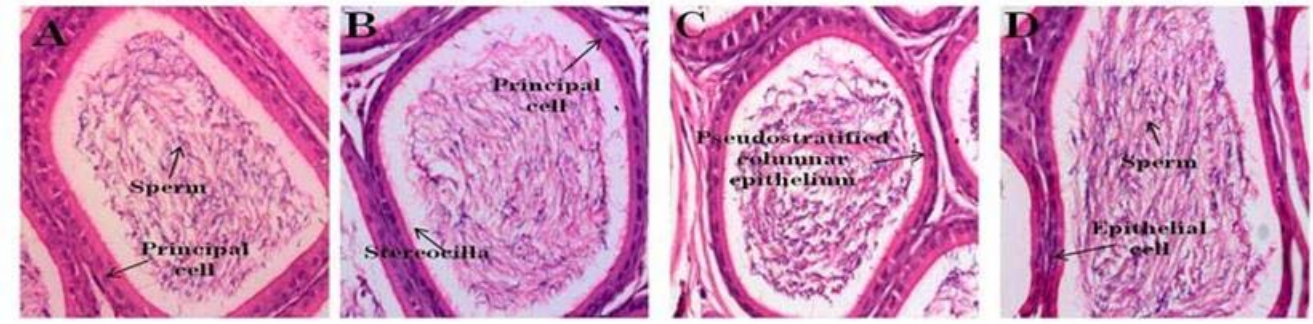

Figure 6: Cross sections of rat's epididymis (H & E staining, 20X). (A): Control, (B): 125 mg/kg, (C): 250mg/kg and (D): 500 mg/kg showed normal sperm density in epididymal lumen.

Histopathological examination revealed intact cells and tissue architectures and no gross pathological lesion in all selected organs of both control and *A. bilimbi* extract treated rats (Figure 3-6).

Acute toxicity study was designed in order to determine the dose that will cause either mortality or serious toxicological effects when given at a single administration dosage. It is also served to provide information on doses that should be used for other toxicity studies, in order to determine long term toxicity effects of the extracts. This includes a longer term studies, for an example in subacute, subchronic or chronic toxicity studies. The OECD 423 Guideline did not directly calculate the precise LD_{50} of extract; however, since death of a proportion of the animals is still the major endpoint of the study, therefore, it is possible to determine the exposure ranges where the lethality is expected.[14] The results from the acute toxicity study indicated that the LD_{50} of the ethanolic extract of *A. bilimbi* is higher than 5000 mg/kg bwt, where it produces neither mortality nor behaviour changes among rats. This finding, therefore, suggests that the extract at the limit dose is essentially non toxic.

In sub acute toxicity study, 125 mg/kg, 250 mg/kg and 500 mg/kg doses were selected. All dosages of 80% ethanolic extract of *A. bilimbi* used did not produce any marked changes among experimental groups of rats, as evidenced by the absence of mortality, toxic symptoms and no changes in body weight gain in both gender.

Analysis of blood parameters is relevant to risk evaluation as the changes in the haematological system give a higher predictive value for human toxicity, when the data is translated from animal studies.[15] Blood is the main medium of transport for many drugs and xenobiotics in the body, therefore, it is often exposed to significant concentrations of toxic compounds.[16] In haematological

evaluation, statistically significant differences were observed in PCV and MCHC with 250 mg/kg dose among male treated rats. Female treated rats showed significant differences in PCV and MCHC values for dose of 250 mg/kg, and significant increased in Hb value for 500 mg/kg dose, as compared with control group. Although the haemoglobin, PCV and red blood cell indices (MCV, MCH and MCHC) were helpful in the differential diagnosis of anaemia [17], gross examinations of the skin, eye and mucous membrane of rats did not show any defect and symptom of anaemia.

The macroscopic examination of the selected organs that included liver, heart, lung, kidney and reproductive organs revealed no prominent changes in colour and texture when compared with control group. This result was supported by histopathological analysis, where it showed normal histological features and absence of any gross pathological lesion at all the doses tested. The cellular morphology, nuclear characteristics and tissue integrity of organs of the treated groups were comparable to the control group.

CONCLUSION

The results had demonstrated that the ethanolic extract of *A. bilimbi* possessed the lowest toxicity effects as revealed in our rat model. No mortality or treatment related changes were observed among the rats that received the highest oral acute of single dose of 5000 mg/kg, and all the subacute tested dosages. Hence, this finding establishes the scientific evidence for the short term and long term safety consumption of *A. bilimbi*.

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