



Preliminary *In vivo* evaluation of the acute toxicity of *Dillenia philippinensis* (Rolfe) fruit extract, Anthocyanins and Polyphenols in Mice (*Mus musculus*)

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Abstract

Dillenia philippinensis (Rolfe) is an endemic plant to the Philippines. Up to date, there are no available data about the toxicity of the fruit extract, anthocyanins and polyphenols from *D. philippinensis* fruits. Therefore, this study investigated their adverse effects in mice. A total of 63 female ICR mice were administered with sterile water (control), crude fruit extract and various doses of anthocyanin and polyphenol at 300, 630, 1000, 2000, 3000, 4000 and 5000 mg/kg. Toxicological observations were completed for 14 days according to the guidelines set by OECD. The signs of toxicity observed were decreased motor activity, poor startle reaction, excessive micturition, decreased respiratory depth and rate, tail lashing and body tremor. Liver abnormalities such as calcification along the portal tract and focal periportal necrosis in the liver of mice treated with 5000 mg/kg anthocyanin while periportal degenerative change (focal and extensive) manifested by loss of cytoplasmic eosinophilia in mice administered with polyphenol at the same dose level confirm their toxicity. Based on the observed clinical signs and histopathological evaluation, the fruits are safe to eat as raw foods. The single oral dose of anthocyanin and polyphenol at 5000 mg/kg is toxic however, 300 mg/kg dose is recommended for daily intake.

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Introduction

Numerous studies reveal that anthocyanins and polyphenols from different fruits possess strong antioxidant activities (Duan *et al.*, 2007; Tan *et al.*, 2011; Muhammad and Akhtar, 2012). Worldwide, consumption of wild fruits is widely promoted due to their rich antioxidant content and higher protective effects against degenerative diseases compared to cultivated and commercial fruits (Rawat *et al.*, 2011; Yildiz *et al.*, 2010).

Dillenia philippinensis (Rolfe), belonging to family Dilleniaceae, popularly known as 'palali', is an endemic tree to the Philippines. It is found throughout the country (World Conservation Monitoring Centre, 1998). The fruits are rounded, soft, fleshy and juicy.

The antioxidant activity of *Dillenia* was reported in several researches. Deepa and Jena (2011) revealed that *D. indica* bark has antioxidant property. Shendge *et al.* (2011) found out that the leaf extracts of *D. indica* exhibit good DPPH, hydroxyl and hydrogen peroxide radical scavenging activity.

Unfortunately, limited toxicological studies involving the fruit extract and its phytochemicals such as anthocyanins and polyphenols have been done (Mennen *et al.*, 2005; Patel *et al.*, 2008; Wattanathorn *et al.*, 2012). Therefore, this study aimed to assess the risk of harm and safe use of the fruits and these compounds in mice through the observation of clinical signs, gross morphology and histopathological changes in the liver and kidneys. Thus, this research may serve as a preliminary basis for the development of new products such as natural organic supplements using the fruit.

Materials and methods

Fruit collection and extraction

The fresh ripe fruits of *D. philippinensis* were collected from Poblacion, Sablan (16°30'13" N 120°28'59" E) in Benguet from September 2013 to February 2014. Randomly selected fruits were packed using zip lock plastic bags and stored into an ice box.

Identification was carried out using keys, online plant databases and taxonomic references. The plant sample was authenticated by Dr. Teodora D. Balangcod, Faculty-In-Charge at the Northern Luzon University Herbarium of the University of the Philippines-Baguio. Voucher specimen was deposited at the Fr. Gerard Braeckman Museum of Natural History in Saint Louis University, Baguio City.

The fruits were washed initially using running tap water to remove dirt and other debris then washed with distilled water. After which, 50 g fruits were sliced into pieces and homogenized for 5 minutes using a blender (Kyowa 1000) with 100 mL acidified methanol (Merck, Germany). Homogenate was soaked for an hour and filtered using Whatman No. 2 by vacuum suction to obtain the filtrate. Aqueous extract was collected by evaporating methanol using the rota-evaporator (Laborota 4011, Heidolph) under 300 mm Hg at 80 ° C and stored at 4 ° C.

Isolation and purification of anthocyanins and polyphenols

The procedures were performed according to the method of Rodriguez-Saona and Wrolstad (2001) using Solid Phase Extraction. A C18 cartridge (Supelco Supelclean ENVI 57064, USA) was conditioned by passing two column volumes pure methanol through the sorbent bed. Methanol was removed by allowing three column volumes acidified deionized distilled water (Merck, Germany) to pass through the cartridge. Approximately 5-10 mL aqueous extract was forced through the cartridge. Afterwards, two column volumes acidified water was used to remove compounds not adsorbed (e.g. sugars, acids). Polyphenols (procyanidins, phenolic acids and flavonols) were collected by adding two column volumes ethyl acetate to the cartridge. On the other hand, anthocyanin pigments were eluted with acidified methanol. Both were transferred and collected in separate flasks. Rota-evaporation was carried out using rota-evaporator (Laborota 4011, Heidolph) to remove the methanol from anthocyanin extract and ethyl acetate from polyphenol extract at 40 ° C and 77 ° C respectively until syrupy. Purified anthocyanin and polyphenol were contained in tubes covered with aluminum foil and stored at 4 ° C.

Dose preparation and administration

The dose was prepared shortly prior to administration. Sterile water for injection (Best Drug Industries, Inc.) was used as a control and solvent or vehicle to dissolve anthocyanin and polyphenol (Castro-Bernas *et al.*, 2005). All animals were fasted from food but not water for 16 hours prior to the single dose tests involving various doses of anthocyanin and polyphenols at 300, 630, 1000, 2000, 3000, 4000 and 5000 mg/kg. Oral gavage was done according to the Institutional Animal Care and Use Committee (2014) standard protocol.

Test animals

Healthy, randomized female ICR albino mice (about 18-24 g/ 4 week old) were obtained from the Research Institute for Tropical Medicine, FILINVEST Avenue, Alabang in Muntinlupa City. All the female mice were nulliparous and non-pregnant. Acclimatization of the animals was done for 7 days prior to dosing based from the studies conducted by Akanmu *et al.* (2004) and Beis *et al.* (2005). The animals were observed for ill health. Animals demonstrating signs of spontaneous disease or abnormality prior to the start of the study were eliminated. The test animals were maintained at standard conditions of 12h light/darkness, humidity and temperature (24 °C ±2 °C) in the Laboratory Animal Housing Facility of the School of Natural Sciences in Saint Louis University, Baguio City. All mice were fed with standard pellets (Robina Starfeeds) and water (Wilkins) *ad libitum*. Animals were randomly selected and group-caged by dose levels (300, 630, 1000, 2000, 3000, 4000, 5,000), and control. They were marked to permit individual identification using different permanent colored markers.

Acute toxicity test

The acute toxicity test of the anthocyanin and polyphenol from *D. philippinensis* fruits in ICR female mice was done in three to four replicates according to the OECD (2002) guidelines 423 with animal research permit (Reference No. AR 2010-108) and approval of the Bureau of Animal Industry.

A total of 63 female ICR mice were randomly distributed to the control and treatment groups. Observations of clinical signs were done within 4 hours after dose administration and at least once per day for a total of 14 days following the directions in Table 1.

Corresponding scores based on the observation made were recorded accordingly. During the observation period, food and water were withheld.

Additional tests involving single dose treatment of 5000 mg/kg anthocyanin and polyphenol and daily dose treatment of 300 mg/kg anthocyanin/polyphenol for 14 days were conducted using Table 2. Scores were interpreted as follows: total score of 0 to 4 is good indicating normal and healthy status of the animal, 5 to 8 is fair while a total score equal to or greater than 9 indicates poor or ill (unhealthy/abnormal) status.

Gross necropsy and histopathological evaluation

The post mortem procedures which involved observation of the gross morphology of organs *in situ* with the naked eyes and collection of key organs (liver and kidney) and tissue samples were done according to the necropsy and sampling procedures by Fiette and Slaoui (2011) and Pines City Doctor's Hospital. Liver and kidney tissue sections were stained using standard Hematoxylin and Eosin staining procedures. Slides prepared were viewed and examined under the microscope (Nikon Eclipse E 100, Japan) with a flexicam (Coolpix Nikon, Japan) at 100x and 400x magnification. Dr. Michael Mostales, a pathologist, was blinded to the treatment when he performed the histological assessments of the slides in triplicates.

Statistical treatment

One-way analysis of variance (ANOVA) at 0.01 level of significance and Post-hoc Tukey test were used to analyze the data. All the experimental results were expressed as the mean and standard deviation. All statistical tests were performed using SPSS 20.0 for Windows software package.

Results

Observed Clinical Signs on the Acute Toxicity Test of Anthocyanin and Polyphenol

Fig. 1 shows that increase in body weight, startle reaction and decrease in motor activity were observed

in all the dose levels of anthocyanin except for the decrease in respiratory depth and rate which were noted in 4000 mg/kg (93.33 ± 10.69) and 5000 mg/kg (74.33 ± 4.16 breaths/ minute) with an average clinical score of 0.67 and 2 respectively.

Table 1. Basic pharmacological signs of toxicity (Castro-Bernas *et al.*, 2004).

Signs of Toxicity	Directions	Clinical Sign Score (Scoring based on the corresponding observations)	
1. Decrease in motor activity	Make noise around the cage for a second or two.	(0)	moved occasionally and spontaneously;
		(1)	did not move spontaneously, but moved slowly when handled;
		(2)	moved sluggishly when handled;
		(3)	did not move at all even when handled.
2. Respiratory rate and depth	Count the number of breaths each mouse takes for a minute. Compare test group with control.	(0)	moderate decrease;
		(1)	definite decrease;
		(2)	pronounced decrease.
3. Startle reaction	Give a strong clap.	(0)	mouse gave a mild start;
		(1)	mouse visibly jerked;
		(2)	mouse jerked, jumped and made sudden frantic attempt to escape.
4. Salivation	Wipe a filter paper beneath the jaw of the mouse. Observe for wetness.	(0)	filter paper remains dry;
		(1)	jaws and chin fur were observed to be wet;
		(2)	saliva actively drips down from the jaws.
5. Micturition	Place filter paper on the floor of the cage. Notice any spots of urine on the paper.	(0)	wet spots observed in the filter paper;
		(1)	upper part of the animal's hind legs are wet.
6. Diarrhea	Place filter paper on the floor of the cage. Notice any change in the consistency of feces.	(0)	soft consistency compared to normal feces;
		(1)	feces spread as it comes out from the anus;
		(2)	feces watery in nature.
7. Circling motion	Observe if the mouse moves around in circles as if reaching for its tail.	(0)	absent;
		(1)	present.
8. Tail lashing	Strike the back of the mouse downward towards the tail. Observe the response on the tail end.	(0)	no response
		(1)	tail lashes from side to side
9. Body tremor	Place the fingers on the back of the mouse. Observe tremor reactions.	(0)	equivocal presence of tremors;
		(1)	definite but sporadic;
		(2)	continuous tremors;
			pronounced tremors nearly clonic convulsions;
10. Death	Record the date and time of death	•	absent;
		•	present.
11. Body weight	Determine the body weight prior to dose administration and weekly thereafter (2 weeks)	(0)	no change in body weight;
		(1)	increase/ decrease in body weight was noted.

The higher score of 5000 mg/kg indicates that the mice treated with this dose experienced more difficulty in breathing than the animals in 4000 mg/kg dose compared to the control (105 ± 6.24 breaths/minute).

On the other hand, the highest average clinical sign score for decrease in motor activity was recorded in 5000 mg/kg dose (2) and lowest in 630 mg/kg (0.67). All the anthocyanin treated mice especially with 5000 mg/kg dose were observed to be inactive and sitting in one corner.

Meanwhile, low startle reaction scores recorded in mice treated with 4000 (1.3) and 5000 (1) mg/kg indicate decreased reaction to external stimulus compared to the others.

Based on the average total clinical sign score, the group administered with the highest dose of anthocyanin (5000 mg/kg) had the highest score (6)

while the lowest dose (630 mg/kg) obtained the lowest score (3.6 ± 0.58) (Fig. 2).

Results obtained provided significant differences in the average total clinical sign scores in the mice administered with anthocyanin except, between and among control, 630, 1000, 2000, 3000 and 4000 mg/kg ($F(6,14) = 53, p < 0.01$).

Table 2. Clinical signs and their corresponding scores (Morton, 1997).

Signs/Severity Score	0	1	2	3
Activity	normal	isolated, abnormal posture	huddled/inactive or overactive	Moribund or fitting
Alertness/Sleeping	normal	dull or depressed	little response to handling	unconscious
Body Condition	normal	thin	loss of body fat, failure to grow	loss of muscle mass
Body Weight	normal weight and growth rate	reduced growth rate	chronic weight loss (>15%) or failure to grow	acute weight loss (>10%), chronic weight loss (20%) or failure to grow & weight loss
Breathing	normal	rapid, shallow	rapid, abdominal breathing	laboured or irregular
Appearance	normal	coat rough/ruffled	unkempt, wounds, hair thinning	bleeding or infected wounds, or severe hairloss or self mutilation
Feces	normal	feces moist	loose, soiled perineum or abnormally dry (+/-) mucus	running out on handling or no feces for 48 hours or frank blood on feces
Movement/gait	normal	slight incoordination or abnormal gait	incoordinated or walking on tiptoe or reluctance to move	staggering or limb dragging or paralysis
Urine	normal		abnormal color/ volume	no urine in 24 hrs or incontinent, soiled perineum

The score of 5000 mg/kg dose is significant (6). Therefore, among the dose levels of anthocyanin, 5000 mg/kg exhibited acute toxicity.

Fig. 3 shows that in terms of motor activity, similar results in the mice treated with 5000 mg/kg anthocyanin were obtained for the 4000 and 5000 mg/kg dose levels of polyphenols (2).

Lack of activity and sitting in one corner were also observed. Only these doses produced mild/poor startle reaction (0), tail lashing (1) and body tremors (3).

In respiratory depth and rate, 5000 mg/kg had the highest score (2) signifying difficulty in breathing (77.5 ± 19.05 breaths/minute).

Despite these clinical signs observed, similar to the single oral administration of anthocyanin doses, there was an increase in the body weight of all the test animals after 14 days.

Fig. 4 shows that 5000 mg/kg (9) followed by 4000 mg/kg (8.67 ± 0.58) dose of polyphenol had the highest average total clinical sign scores while the lowest was in 630 mg/kg (3.33 ± 0.58).

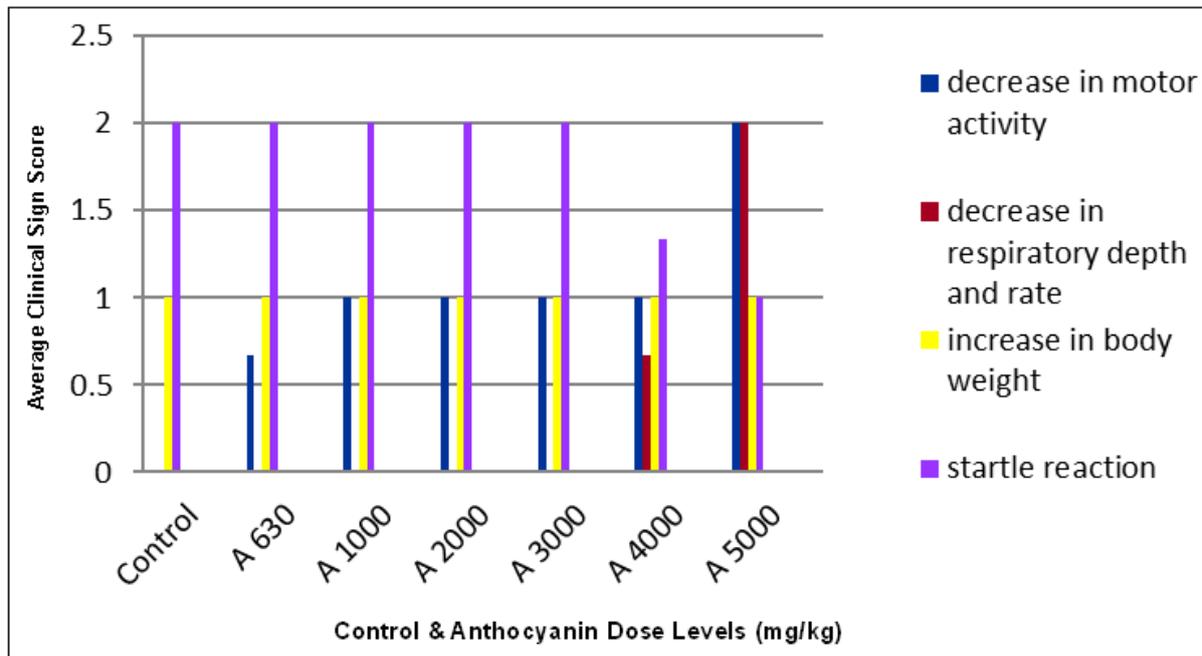


Fig. 1. Average scores of clinical signs observed after single administration of anthocyanin dose compared to control.

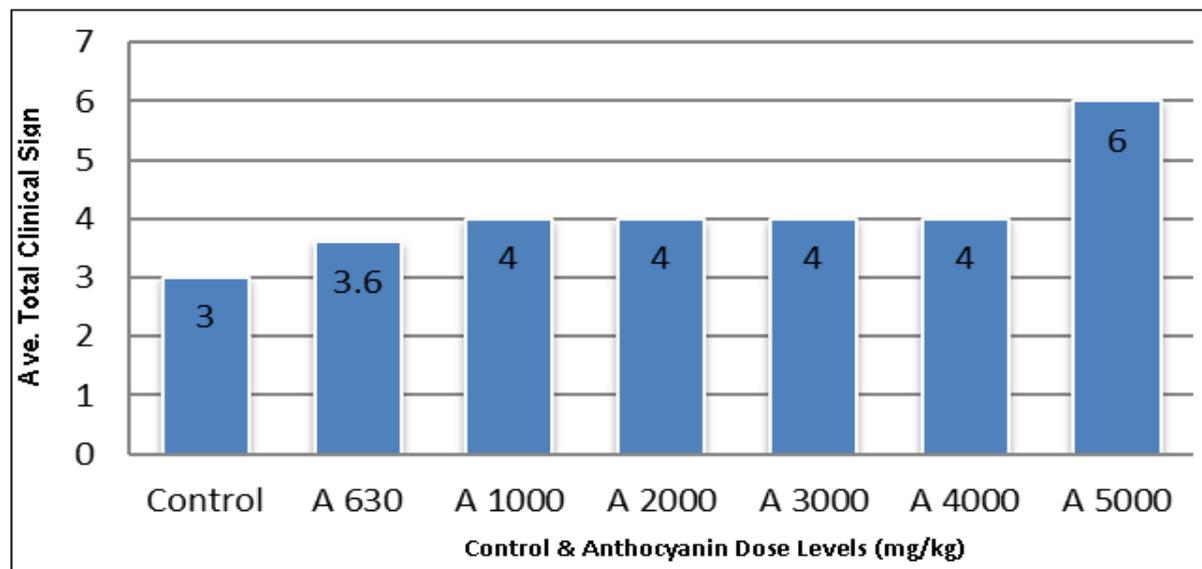


Fig. 2. Average total clinical sign score of anthocyanin dose levels compared to control.

*Significant difference ($p < 0.01$).

The clinical signs produced by 4000 and 5000 mg/kg are significantly different from the other dose levels and control, except between these two doses ($p < 0.01$). Therefore, these doses significantly exhibited clinical signs of acute toxicity. On the other hand, the clinical signs observed in mice treated with 630, 1000, 2000 and 3000 dose levels compared to the control do not significantly differ between and among these treatments.

Fig. 5 reveals that only the 5000 mg/kg dose of anthocyanin and polyphenol produced clinical signs indicating toxicity. Fig. 6 shows significant average total clinical score signs between anthocyanin and polyphenol at 5000 mg/kg ($p < 0.01$). From the data obtained, the average total clinical sign score for polyphenol (13.25) is higher than anthocyanin (10.25). This signifies that polyphenol dose is more toxic than anthocyanin.

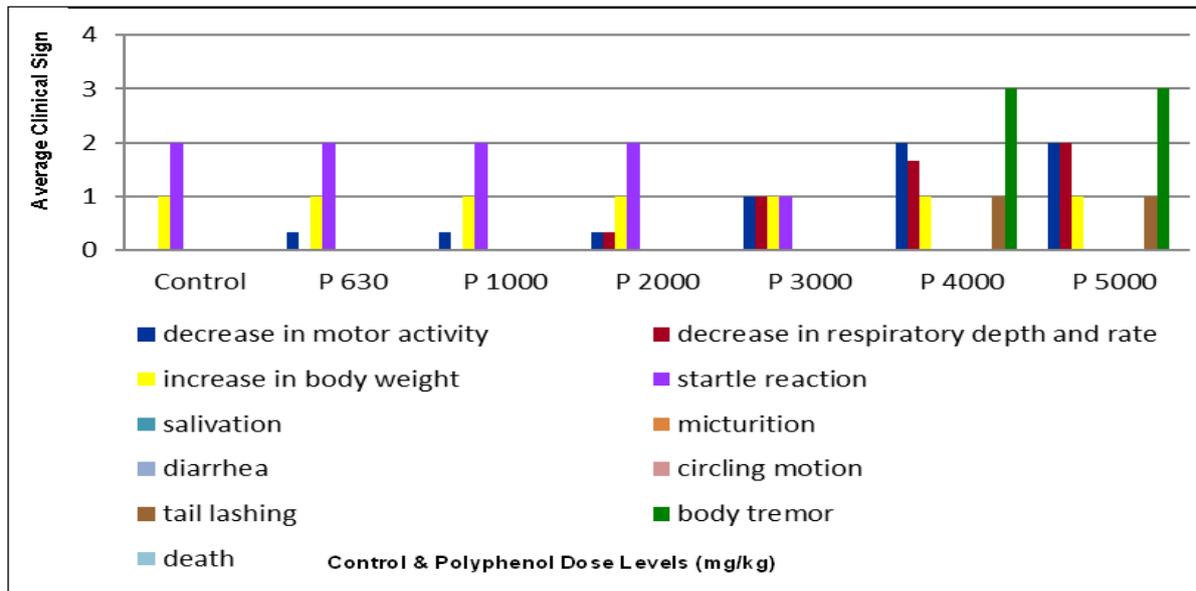


Fig. 3. Average of clinical sign scores observed after single administration of polyphenol dose compared to control.

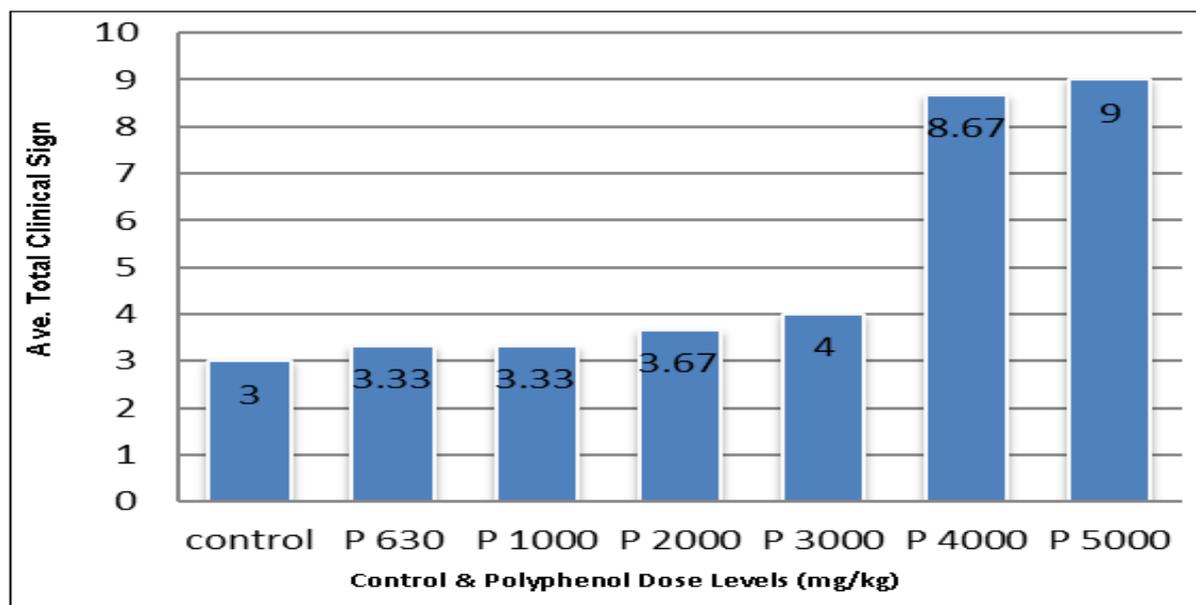


Fig. 4. Average total clinical sign score of polyphenol dose levels. *Significant difference ($p < 0.01$).

In this study, the crude extract and 300 mg/kg dose of anthocyanin and polyphenol are the same as the control which are non toxic (0).

Gross necropsy on test animals and histopathological evaluation

Histopathological tests were conducted to evaluate the acute toxicity of the crude extract, anthocyanin and polyphenol at 300 and 5000 mg/kg. Necropsy of all the test animals and evaluation of the kidney and liver tissues were carried out in at least three

replicates to confirm whether the internal organs had been damaged.

Macroscopic observation of the kidneys and liver revealed no deviation from the normal state (Fig. 7). The results showed no gross pathological changes or abnormalities in all the treated mice compared to the control. The liver and kidneys occupied the right location or position, exhibited the normal shape, consistency, size and color.

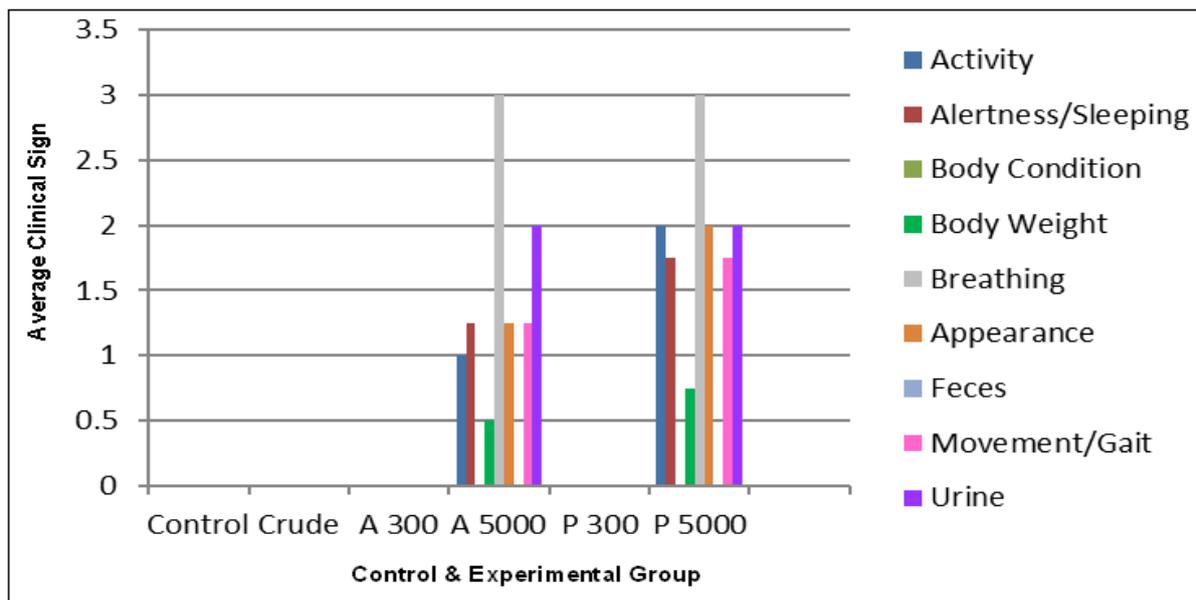


Fig. 5. Average scores of clinical signs observed in the crude extract and selected doses of anthocyanin and polyphenol compared to control.

The bean shaped kidney was brownish red while the liver was dark reddish, hence, normal. The right kidney was larger than the left kidney. It was more cranially located and quite heavier. These characteristics are also normal. The kidneys were surrounded by a capsule.

The histology of the kidney and liver from mice treated with crude fruit extract, anthocyanin and polyphenol at 300 mg/kg compared to the control can

be described as ‘histomorphologically unremarkable’ which indicates normal or healthy state (Fig. 8-9).

The sections observed showed tubular and glomerular structures in kidney while hepatocytes, bile ducts, portal triads and central veins in the liver that were all normal. The results suggest that the crude fruit extract and 300 mg/kg anthocyanin and polyphenol do not have adverse microscopic effects. Thus, are safe and practically non-toxic.

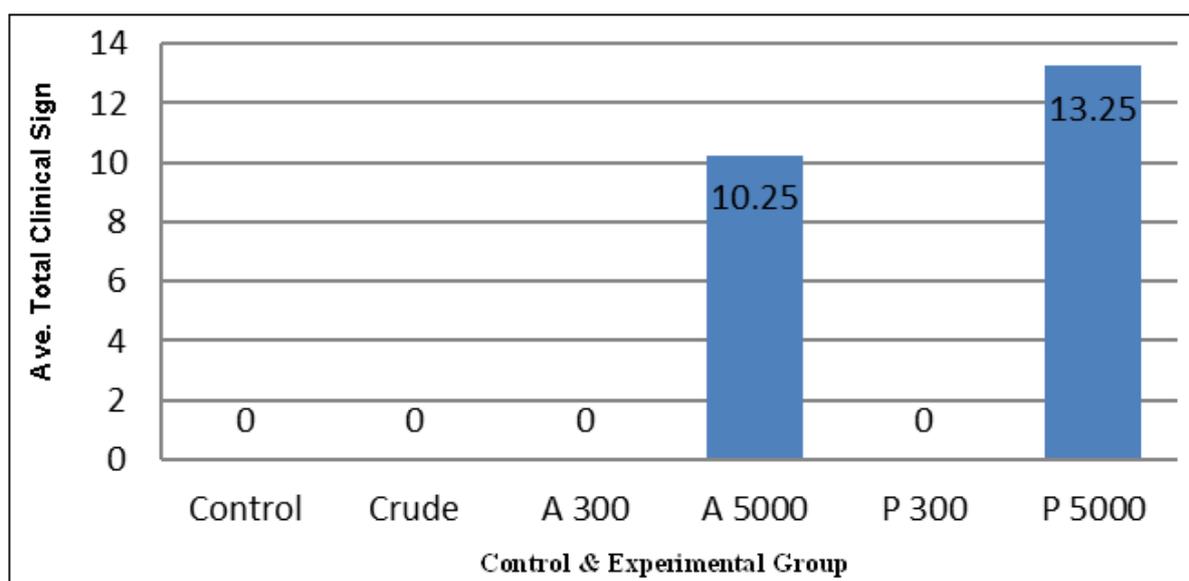


Fig. 6. Average total clinical sign scores in the crude extract and selected dose levels of anthocyanin and polyphenol compared to control. *Significant difference ($p < 0.01$).

Although the gross necropsy results showed no macroscopic changes, the histologic sections of the liver from mice treated with a single oral dose of anthocyanin and polyphenol dose at 5000 mg/kg revealed abnormalities (Fig. 10). Several foci of calcification along the portal tract and focal periportal necrosis were observed in mice treated with anthocyanin at 5000 mg/kg. In this study, calcification was manifested by the thickening of layers in the portal vein and presence of amorphous

calcium deposits while necrosis was manifested by the swelling of hepatocytes and nuclear disintegration. On the other hand, periportal degenerative change (focal and extensive) manifested by loss of cytoplasmic eosinophilia was noted in mice orally administered with polyphenol at 5000 mg/kg. These microscopic findings suggest that the highest dose of anthocyanin and polyphenol (5000 mg/kg) induce acute toxicity in mice.

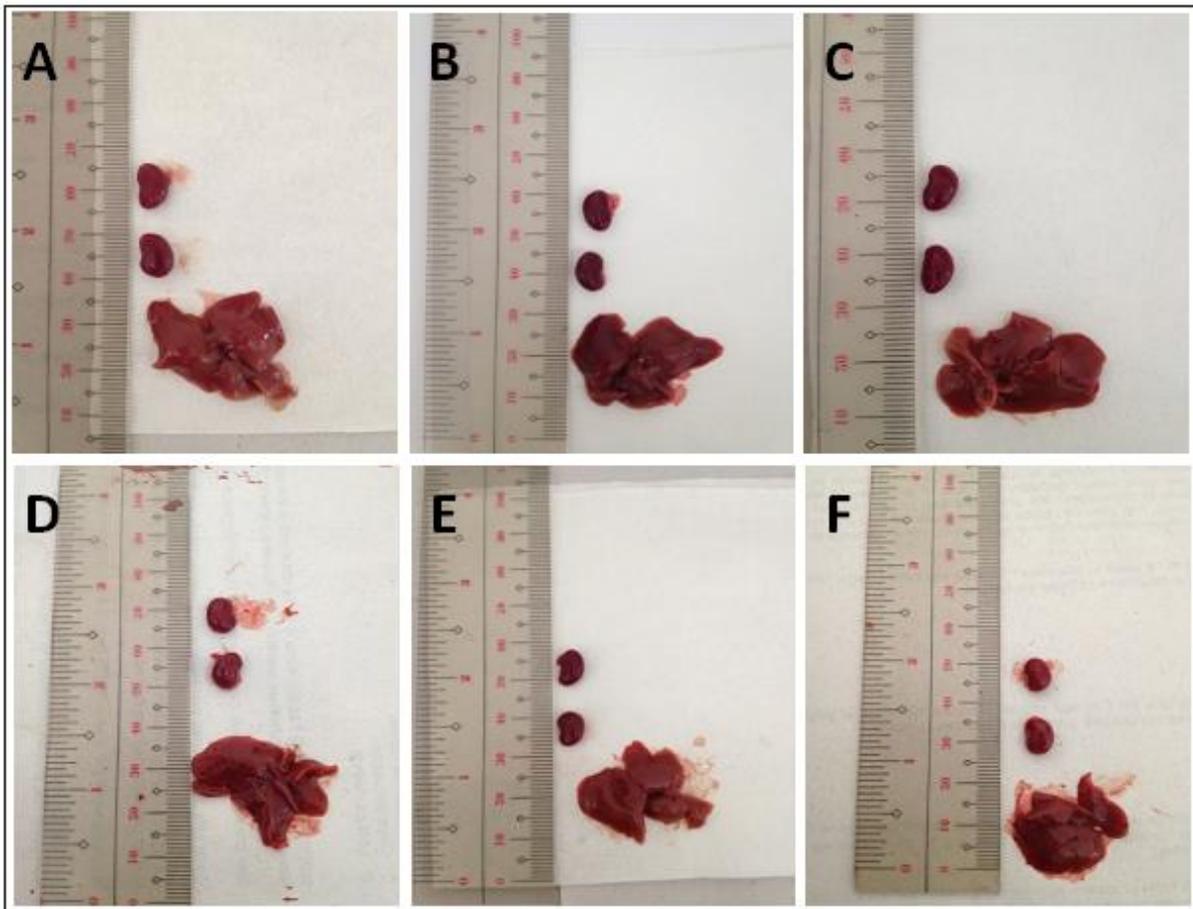


Fig. 7. Gross observation of kidneys and liver from mice treated with crude fruit extract, anthocyanin and polyphenol (300 mg/kg & 5000 mg/kg) compared to control (A= control, B= crude, C= 300 mg/kg anthocyanin, D= 5000 mg/kg anthocyanin, E= 300 mg/kg polyphenol, F= 5000 mg/kg polyphenol).

Discussion

Observed Clinical Signs on the Acute Toxicity Test of Anthocyanin and Polyphenol

Inactivity, poor startle reaction, tail lashing, body tremors and difficulty in breathing are indicators of toxicity in mice (Gatsing *et al.*, 2010; Singh *et al.*, 2012). According to McGill Comparative Medicine and Animal Resource Centre (2016), the normal

number of breaths in mice is 95-165 per minute. This signifies that the high doses of anthocyanin and polyphenol especially 5000 mg/kg result to respiratory depression. Poor startle response as a central nervous system reflex indicates toxicity (Schaeffer, 1993). In this study, it can be noted that as the dose increases, the degree of response to stimulus by the treated mice decreases.

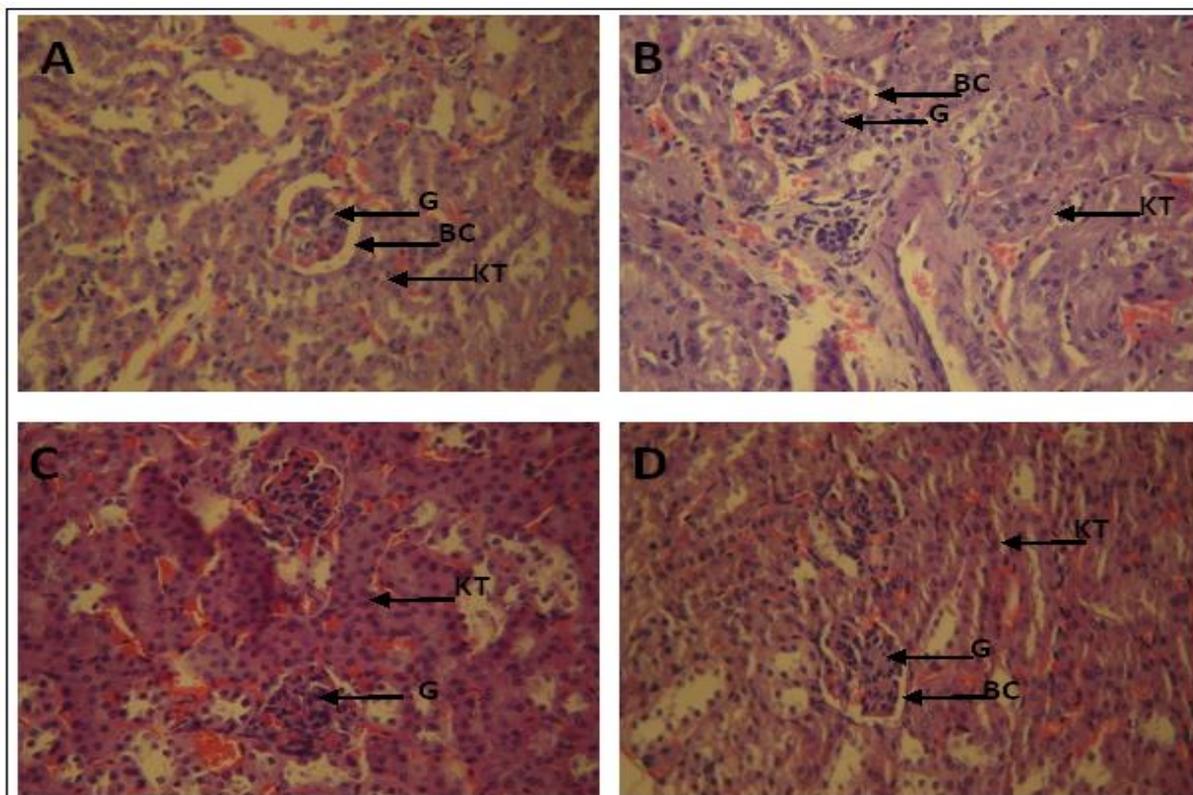


Fig. 8. Photomicrographs showing representative kidney sections from mice treated with crude fruit extract and 300 mg/kg anthocyanin and polyphenol compared to control (A= control, B= crude, C= anthocyanin, D= polyphenol, H & E 400x magnification).

Legend: G- glomerulus, KT- kidney tubules lined by simple cuboidal epithelium, BC- Bowman's capsule.

Moreover, tail lashing and body tremor are normally produced due to high or very high doses of test substances indicating toxicity (National Registration Authority for Agricultural and Veterinary Chemicals, 1997).

According to Sergediene *et al.* (1999), polyphenol antioxidants exhibit a dose- dependent toxicity due to their prooxidant properties which involves formation of reactive species such as malondialdehyde. Halliwell (2008), cited that polyphenols become pro-oxidants under high concentrations and in the presence of transition metals. Moreover, studies report the carcinogenic and genotoxic effects of polyphenols at high doses or concentrations. This may explain the higher toxicity of polyphenols compared to anthocyanins. Weaver *et al.* (2009), revealed that polyphenols (tannins) from *Fragaria ananassa* c.v. Elsanta are more cytotoxic to normal and tumour cell lines or lymphocytes compared to its anthocyanin extract.

Further, Hagiwara *et al.* (1991) observed that caffeic acid (polyphenol) is carcinogenic in rats after feeding them daily with 2 % concentration (20g/kg diet) for 104 weeks.

The doses of anthocyanin (flavonoid) and polyphenol (300 up to 5000 mg/kg) are classified under Category IV of chemical substances which indicates very low toxicity (Lorenz, 2006; EPA, 2012). Similar studies reported no mortality to test animals used. Dunnick and Hailey (1992) fed flavonoid (quercetin) to F344/N rats at 40-1,900 mg/kg dose per day. At the end of their study, they concluded that quercetin is safe as it did not cause death to the test animals. Martin and Appel (2010) cited that the risk for polyphenol toxicity is relatively low in rodents. This is mainly because polyphenols are poorly absorbed in the gut. In the study conducted by Hu (2007), polyphenols had low bioavailability at 2% -20% ($\leq 10\%$ of the ingested dose).

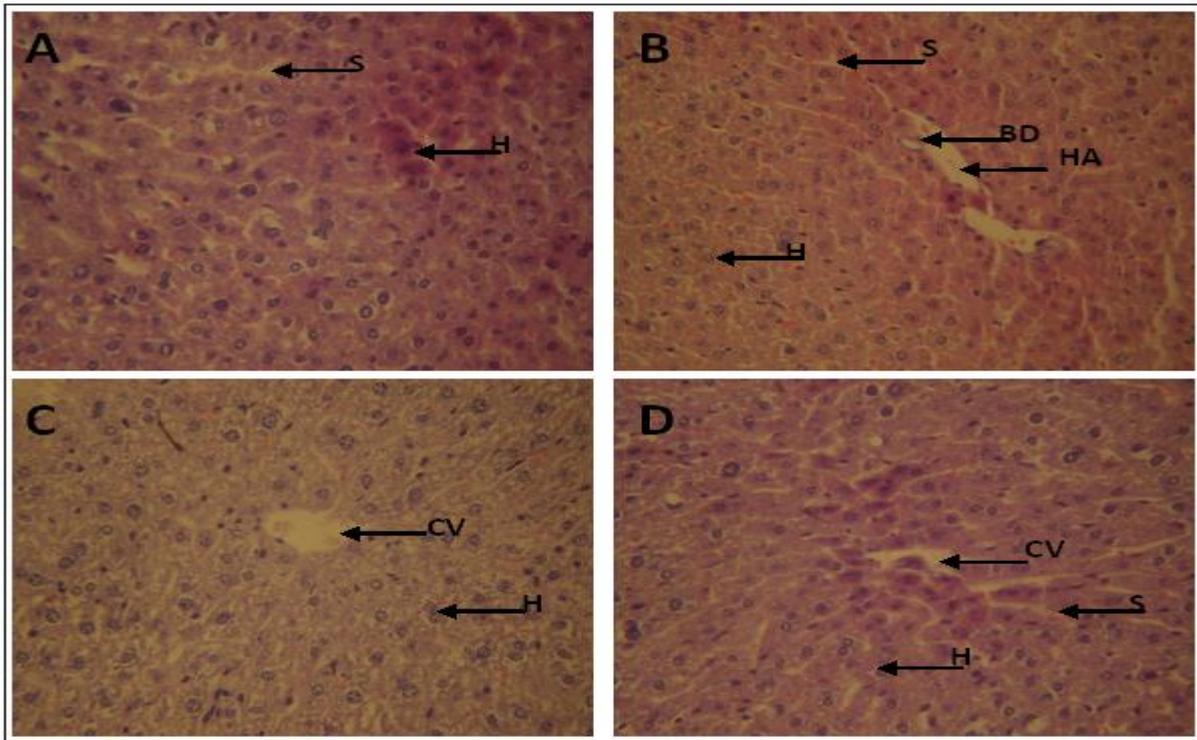


Fig. 9. Photomicrographs showing representative liver sections in mice treated with crude fruit extract and 300 mg/kg anthocyanin and polyphenol compared to control (A= control, B= crude, C= anthocyanin, D= polyphenol, H & E 400x magnification).

Legend: CV- central vein, H- hepatocytes, S- sinusoids, HA- hepatic artery, BD- bile duct.

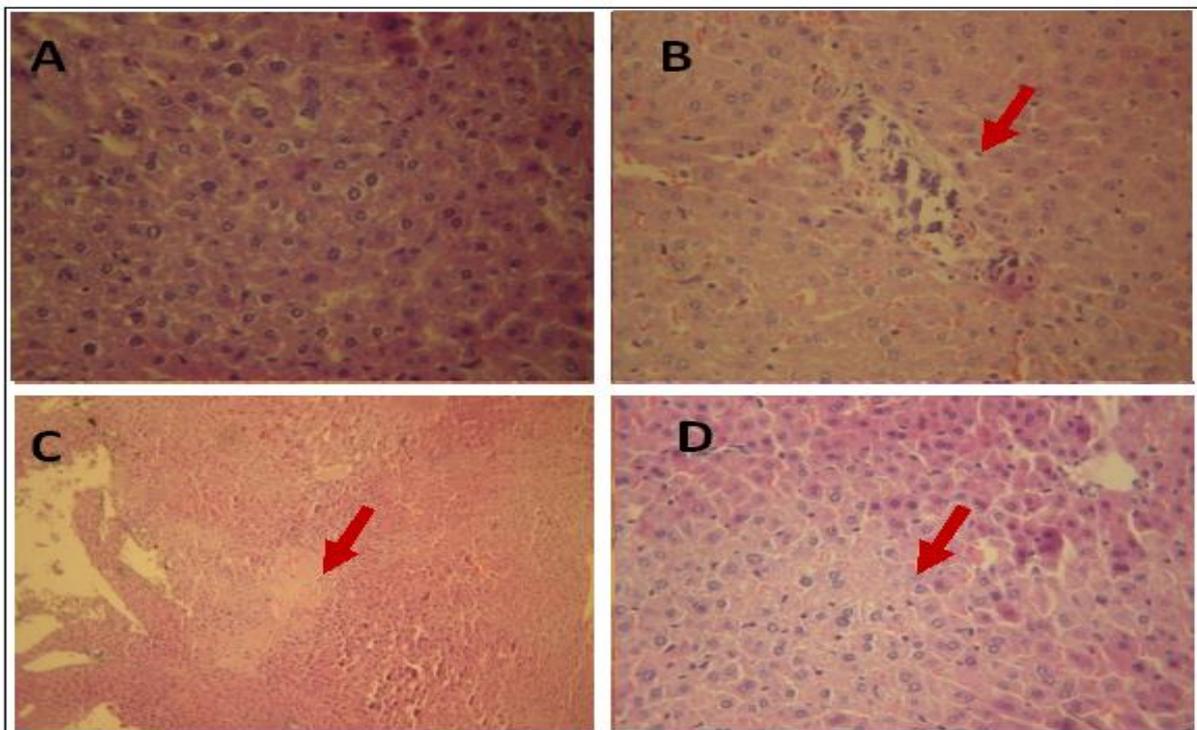


Fig. 10. Photomicrographs showing liver sections with abnormalities in mice treated with 5000 mg/kg anthocyanin and polyphenol compared to control. (A= normal, B= several foci of calcification along the portal tract, C= focal periportal necrosis, D= focal and extensive periportal degenerative change, H & E 400 x magnification).

Solubility, chemical nature and structure of the polyphenol caused slow absorption process since pure aglycones of polyphenols have poor solubility (< 20 µg/ml in water). Further, Ray *et al.*, (2001) reported in their study that there was no acute toxicity noted in the rodents after oral grape seed polyphenol (proanthocyanidin) extract intake at 500-2000 mg/kg dose. Thus, they concluded that the lethal dose for the extract in the test animals was greater than 5000 mg/kg.

Similar to polyphenols, anthocyanins are poorly absorbed (Martin and Appel, 2010). Since these compounds are water soluble pigments, they can easily be removed from the body often through micturition (Prior and Wu, 2006). Apart from this, the low toxicity of this compound may be due to the chemical reactions it undergoes in the gut especially in the liver where detoxification takes place. Flavonoids (epicatechin) consumed by rats had undergone conjugation reactions that involved glucuronidation in the intestinal mucosa, sulfation in the liver, and methylation in the liver and kidney to increase its circulatory elimination time (Piskula and Terao, 1998; Nijveldt *et al.*, 2001). These studies support the very low toxicity of anthocyanins due to its low bioavailability.

Gross necropsy on test animals and histopathological evaluation

Necrosis and degeneration of hepatocytes signify the occurrence of death in the liver cells (Cotran *et al.*, 1994; Klaassen and Watkins, 2010). Calcification or abnormal hardening in the liver due to the deposition of calcium salts indicates liver damage (Conway, 2000; Khan *et al.*, 2013). Upon oral ingestion, polyphenols and anthocyanins are hydrolyzed, absorbed and metabolized in the body.

In the liver, these compounds undergo structural modifications to reduce their toxicity. Conjugation processes such as glucuronidation, sulfation and methylation occur to increase their solubility and molecular weight that contribute to their high elimination time from the body through biliary and urinary excretion (Archivio *et al.*, 2007).

However, these detoxification mechanisms are not enough to prevent cell damage as observed in the study conducted.

Conclusion

Based on the observation of short-term clinical signs, necropsy findings and histopathological evaluation of liver and kidney tissues, only the single oral dose of anthocyanin and polyphenol at 5000 mg/kg is toxic but not lethal to female ICR mice.

This study provides supporting evidence that *Dillenia philippinensis* or 'palali' wild fruits are safe to eat hence, non-toxic. The anthocyanin and polyphenol from the fruit can be a source of natural organic supplements and antioxidants that can increase the overall health status of the body. Thus, 300 mg/kg is the recommended daily dose intake to be used in humans.

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