Hepaotoprotective and nephroprotective activities of *Pistacia integerrima* fruit extract in paracetamol intoxicated male rabbits with effect on blood cells count

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Abstract: The beneficial effects of *Pistacia integerrima* (PI) fruit methanol extract on some liver and kidney related parameters and blood cells count of paracetamol (PCM) intoxicated male rabbits were studied. Paracetamol intoxication caused remarkable increase in the serum ALT, AST and ALP levels. The PCM intoxicated rabbits that received PI extract orally at doses of 200 mg and 400 mg/kg b.w. /oral/day for 16 days showed significant reduction in serum ALT, AST and ALP levels ($P<0.05$). Liver microsections from PCM intoxicated rabbits treated with PI fruit methanol extract showed improvement in the liver histoarchitecture. The urine output of PCM intoxicated control rabbits group was significantly lower ($P<0.05$). The PCM intoxicated rabbits that received PI extract showed significant increase in urine output ($P<0.05$). The PCM intoxicated rabbits treated with PI extract also showed significant reduction in the levels of serum urea and creatinine ($P<0.05$). The renal creatinine clearance of PCM rabbits treated with PI extract improved significantly ($P<0.05$). Microsections of kidneys from PCM intoxicated rabbits treated with PI fruit methanol extract showed improvement in renal histoarchitecture. During this study, PI extract caused no improvement in the RBC count of PCM intoxicated rabbits. However, the extract caused significant increase in WBC and platelets count ($P < 0.05$) of PCM intoxicated rabbits. From the findings of the present research, it was concluded that oral administration of *P. integerrima* fruit methanol extract is beneficial for the liver and kidney related biochemical parameters and blood cells count of paracetamol intoxicated male rabbits.

Keywords: ALT, AST. ALP, creatinine, urea, urine output, creatinine clearance, blood cells count.

INTRODUCTION

Paracetamol (PCM) is one of the most commonly used analgesic drugs and its overdosing results in liver injury (Marks *et al*., 2017). The detail of mechanism involved in paracetamol induced liver injury is much complex and many extracellular and intracellular processes are involved in this injury, which include paracetamol metabolism, autophagy, oxidative stress of mitochondria and endoplasmic reticulum, liver regeneration, sterile inflammation and dysfunction in microcirculation (Yan *et al*., 2018). During its metabolism, a metabolite known as *N*-acetyl-p-benzoquinonimine (NAPQI) is produced which is eliminated from the body after its conjugation with glutathione (GSH). In case of excessive production of NAPQI due to PCM overdose, GSH is not sufficient to detoxify NAPQI (Vermeulen *et al*., 1992). NAPQ1 binds with macromolecules on cellular membranes through covalent bond which leads to tissue necrosis and ultimately organ dysfunction (Bessems and Vermeulen, 2001). Necrosis of liver tissues results in the increased leakage of liver function enzymes such as alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT) into the blood stream (Sabir and Rocha, 2008). Hepatotoxicity due to paracetamol overdosing is a global issue, and in the USA, paracetamol overdosing accounts for more than 50% of overdose-related acute liver failure (Yoon *et al*., 2016). Massive paracetamol overdose keep the patients at high risk of organ damage (Marks *et al*., 2017).

Excessive production of NAPQ1 due to paracetamol overdosing also causes renal failure in both humans and animals (Eguia and Materson, 1997). Paracetamol overdosing results in vasoconstriction and inhibition of vasodilation in the kidneys, which in turn result in decreased blood flow to the renal blood vessels and reduced glomerular filtration rate (Whelton *et al*., 2003). Paracetamol overdosing is especially toxic for the kidneys of individuals that suffer from starvation or regularly drink alcohol or take P450 microsomal oxidase enzymes stimulating drug (Okwuosa *et al*., 2009). Recently, Zhu *et al*. (2018) reported that administration of diclofenac and

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PCM in combination to the patient for pain relief after surgery causes acute kidney injury.

There are also reports about the adverse effect of prolonged paracetamol treatment on haematological parameters in mammals. For example, treatment of female albino rats with paracetamol at oral dose of 7.5 mg/kg body weight caused reduction in RBC count, hemoglobin (Hb) concentration, packed cell volume (PCV) value, total WBCs, neutrophil, eosinophil, monocyte, lymphocyte and platelet count (Oyedeji *et al*., 2013). The treatment of PCM induced hepatotoxicity and nephrotoxicity has toxicological importance. Some antioxidants like vitamin C, Vitamin E and melatonin have been used for the treatment of PCM induced hepatotoxicity in mice (Sener *et al*., 2003). Natural products from plant origin are believed to be an important source of therapeutic agents (Calixto, 2005). Plant based medicines are safer than synthetic medicines (Vongtau *et al*., 2005). Medicinal plants are rich source of pharmaceuticals for the treatment of different human ailments due to their possession of diverse phytochemicals and broad biological functionality (Jaroszewski *et al*., 2005). The protective effects of medicinal plants on liver and kidney of paracetamol intoxicated animal models have been reported (Murad *et al*., 2015; Saleh *et al*., 2018).

Pistacia integerrima (Stewart) is a deciduous tree and belongs to the family Anacardiaceae. It is found in East Afghanistan, Pakistan, and North West and West Himalaya at an altitude of 800-1900 m (Pant and Samant, 2010). Traditional medical practitioner uses the bark and fruit of this tree for the treatment of liver diseases (Ahmad *et al*., 2014). The plant has been reported for pharmacological activities including antimicrobial, antioxidant, analgesic, cytotoxicity and phytotoxicity (Bibi *et al*., 2015). The aim of the present study was to assess the beneficial effects of methanol extract of *P. integerrima* fruit on the liver, kidney and hematological parameters of paracetamol intoxicated male rabbits.

MATERIALS AND METHODS

Plant materials

Dry fruits of *P. integerrima* (PI) were purchased from trader of medicinal plants at Mingora city, Swat, Pakistan. The fruits were identified by taxonomists in the Department of Botany, University of Malakand, Khyber Pakhtunkhwa. The PI fruits were ground in electric chopper to get fine powder form. For extract preparation, 450 grams PI fruit powder was soaked in 96% methanol (2000 ml) for three days. During soaking, the plant material was shaken occasionally and then filtered through filter paper (Whatman no. 42). The filtrate was evaporated through vacuum rotary evaporator (Heidolph Laborta 4000 efficient). Finally a brown colour crude extract in thick paste form was obtained. The weight of extract obtained was 172.5 gram (38.33 % w/w).

Animal grouping and study design

Male domestic rabbits (*Oryctolagus cuniculus*) weighing 800-1000 grams and 5-6 months of age were used during experiment. They were housed in Animal chambers at the campus of University of Malakand. Fresh vegetable including carrots were provided to the rabbits. The rabbits had free access to water. This study was approved by University of Malakand Animal Ethics Committee.

After two weeks of acclimation, the acute toxicity test of PI extract was conducted according to the OECD guidelines no. 420. The extract was found safe up to the highest dose (2500mg/kg body weight) used during this test. After acute toxicity test, a total of 16 healthy male rabbits were selected for experiment. For identity of rabbit groups and individual rabbits in a group, different color tags were applied. Sixteen rabbits were divided into the following four groups (four rabbits in each group): 1) normal control group which received an intraperitoneal injection of 10mL normal saline on day 0 and then treated orally with 5mL normal saline/ day/oral for 16 days, 2) PCM control group which received an intraperitoneal injection of paracetamol (1000 mg/ kg. b.w. in 10ml normal saline) on day 0 and then treated orally with 5mL normal saline/ day/oral for 16 days, 3) PCM rabbit group which received an intraperitoneal injection of paracetamol (1000 mg/kg. b.w. in 10ml normal saline) on day 0 and then treated orally with PI extract at a dose of 200mg/kg b.w. /oral daily for 16 days, suspended in 5ml normal saline, 4) PCM rabbit group which received an intraperitoneal injection of paracetamol (1000mg/kg. b.w. in 10 ml normal saline) on day 0 and then treated orally with PI extract at a dose of 400mg/kg b.w. /oral daily for 16 days, suspended in 5 ml normal saline.

Collection of urine and analysis

On the 16^{th} day of experiments, all the rabbits were kept individually in separate cages which were especially designed for rabbit urine collection. Urine samples were collected for 24 hours. Each rabbit had free access to drinking water during the period of urine collection. The total urine volume of each rabbit collected for 24 hours was measured in milliliter (ml) by using the graduated cylinder. Urine samples were stored at 4^0C and analyzed on next day for assessment of urinary creatinine. Urine creatinine level was estimated through COBAS chemistry automation using Roche Diagnostic kit. The estimations of urine volume and urine creatinine were done for calculation of creatinine clearance.

Blood collection and study of different parameters

After urine collection, the animals were sequentially anesthetized with inhaled diethyl ether. Each rabbit restricted on the dissecting board was dissected and 3 mL blood was collected from the heart ventricle through 5 ml

syringe with 21 Gauge needle and then expelled gradually into Ethylene Diamine Tetra-acetic Acid (EDTA)-coated tubes for hematological analysis. Another 3 ml blood was collected by the same method in sterile tubes with coagulant for estimation of liver and kidney related serum biochemical parameters. These blood samples were then subjected to centrifuge (Shimazu, Japan) at 3000 revolutions per minute for 5 minutes for separation of sera. The sera were placed into labeled eppendorf tubes at room temperature and then assayed for liver related serum parameters i.e., ALT, AST and ALP, and kidney related serum parameters i.e., urea and creatinine. The concentrations of these biochemical parameters were estimated through COBAS chemistry automation using Roche Diagnostic kits. The blood samples collected in EDTA coated tubes were analyzed for estimation of red blood cell (RBC), white blood cell (WBC) and platelets count. The RBC, WBC and platelet count were determined by automated blood cell analyzer (Sysmex Co, Japan). During dissection of the rabbits, liver and kidneys were excised, washed with phosphate buffer and dried with tissue paper and then transferred to 10 percent formalin fixative solution in glass bottles which were labeled accordingly. Within 24 hours the liver and kidney tissues were processed for paraffin embedding and sections of 5-micron thickness were taken by a microtome. The sections were stained with hematoxylin and eosin, slides were prepared and then examined under microscope for histopathological changes and images were captured through attached CCTV camera.

Creatinine clearance (C) study

Creatinine clearance tests are widely used to determine the efficiency of glomerulus in filtration (Rose and Post, 2001). The following method of van Acker *et al*. (1992) was used for the determination of Creatinine clearance:

$$\text{Clearance} = \frac{\text{Urine creatinine } \left(\frac{\text{mg}}{\text{dl}}\right) \times \text{Urine volume (ml) /24 hours/1440 minutes}}{\text{Serum creatinine } \left(\frac{\text{mg}}{\text{dl}}\right)}$$

Data analysis

During this study, the values were presented as means with standard error. Tukey Test was applied for the comparisons of parameters among different rabbit groups. Computer software SPSS 16.0 was used for comparison. Percent changes in parameters of paracetamol group of rabbits were calculated by the following method:

$$\frac{\text{Mean value of a parameter of PCM control group} - \text{Mean value of a parameter of control group}}{\text{Mean value of a parameter of control group}} \times 100$$

Percent changes in parameters between paracetamol control rabbits and paracetamol intoxicated rabbits treated with extract were calculated by the following method:

$$\frac{\text{Mean value of a parameter of extract treated group} - \text{Mean value of a parameter of PCM control group}}{\text{Mean value of a parameter of PCM control group}} \times 100$$

RESULTS

Effects of PI extract on some liver related serum parameters

Table 1 shows the serum levels of some liver related biochemical parameters i.e., ALT, AST and ALP of different rabbit groups. The serum ALT, AST and ALP levels of PCM control rabbit group were 122.5 %, 238.3 % and 59.9 % higher than the normal control rabbit group, respectively. The difference was significant ($P<0.05$). The PCM intoxicated rabbit groups treated at two extract doses (200 mg and 400 mg/kg) showed significant decrease in serum ALT, AST and ALP levels as compared to PCM control rabbit group ($P<0.05$). For example, the serum ALT, AST and ALP levels of PCM intoxicated rabbits treated with 200 mg/kg PI extract were 23.2 %, 20 % and 20.2 % lower than the serum ALT, AST and ALP levels of PCM control rabbit group, respectively. The serum ALT, AST and ALP levels of PCM rabbit group treated with 400 mg/kg PI extract were 39.4 %, 50.7% and 27% lower than the serum ALT, AST and ALP levels of PCM control rabbit group, respectively.

Effect of PIF extract on some kidney related parameters

The effect of PIF extract on some kidney related parameters of PCM intoxicated rabbits is shown in table 2. The urine output of PCM control group of rabbits was 76.04 % lower than the normal control group of rabbits. The difference was significant ($P<0.05$). The PCM intoxicated rabbits treated at two doses of PI extract showed significant increase in urine output as compared to PCM control group ($P<0.05$). For example, the urine outputs of PCM rabbit groups orally treated with 200 mg/kg and 400 mg/kg PI extract were 250.9% and 269.5% higher than the PCM control rabbit group, respectively. The difference from PCM control group was significant ($P<0.05$). The urine output of PCM intoxicated rabbits treated with the 400mg/kg PI extract was insignificantly higher than the urine output of PCM intoxicated rabbits treated with the 200mg/kg extract ($P>0.05$).

The serum levels of urea and creatinine of PCM control group of rabbits were 73.33 % and 333.3 % higher than the normal control group of rabbits, respectively.

The PCM rabbit groups treated with PI extract showed significant decrease in serum levels of urea and creatinine as compared to the serum levels of urea and creatinine of PCM control rabbit group ($P<0.05$). The serum levels of urea and creatinine of PCM intoxicated rabbit group treated with 200 mg/kg PI extract was 32.7% and 61.53% lower than the PCM intoxicated control rabbit group,

Table 1: Effect of PI fruit methanol extract on some liver related biochemical parameters of PCM-intoxicated male rabbit.

Animal groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Healthy control	31 ± 3.9 [a]	30 ±2.9 ±3.0 [a]	64.7 ±2.7 [a]
PCM control	69.0 ±1.8 [d] (+122.5 %)	101.5 ± 7.1 [d] (+238.3%)	103.5 ±4.1 [d] (+59.9%)
PCM+PI extract 200 mg/kg. b. w	53.0 ±2.6 [c] [-23.2 %]	80.6 ±4.1 [c] [-20%]	82.5 ±2.2 [bc] [-20.2%]
PCM+PI extract 400 mg/kg. b. w	41.8 ±2.3 [b] [-39.4 %]	50 ±3.2 [b] [-50.7%]	75.5 ±2.1 [ab] [-27%]

.- means sharing no letter are significantly different at P<0.05 in Tukey test in one way anova, values in parentheses indicate percent increase (+) or decrease (−) in parameters of PCM control rabbit group from normal control rabbit group, values in brackets indicate percent increase (+) or decrease (−) in parameters of PCM rabbit groups treated with PI extract from PCM control rabbit group.

Table 2: Effect of PI fruit methanol extract on urine output and some kidney related biochemical parameters of PCM-intoxicated male rabbits

Animal groups	Urine output (ml/h)	Urea in serum (mg/dl)	Creatinine in serum (mg/dl)	Creatinine clearance (ml/min)
Healthy control	299.7±8.6 [c]	12.0 1.3[a]	0.3 ±0.06 [a]	4.7 +1.4 [d]
PCM control	71.8 ±3.9 [a] (-76.1 %)	20.8±0.9 [b] (+73.33)	1.3 ±0.24 [b] (+333.3 %)	1.78+ 0.3[a] (-62.1 %)
PCM+PI extract 200 mg/kg. b. w	252.0±5.4 [b] [250.9 %]	14.0 1.3 [a] [-32.69]	0.5 ±0.1 [a] [-61.53 %]	2.5 ±0.4[b] [+40.4 %]
PCM+PI extract 400 mg/kg. b. w	269.5±10.2 [b] [274.7 %]	12.3±0.9 [a] [-40.9 %]	0.7 ±0.2 [ab] [-46.15 %]	3.1 ±0.4 [+74.2 %][c]

.- means sharing no letter are significantly different at P<0.05 in Tukey test in one way anova, values in parentheses indicate percent increase (+) or decrease (−) in parameters of PCM control rabbit group from normal control rabbit group, values in brackets indicate percent increase (+) or decrease (−) in parameters of PCM rabbit groups treated with PI extract from PCM control rabbit group

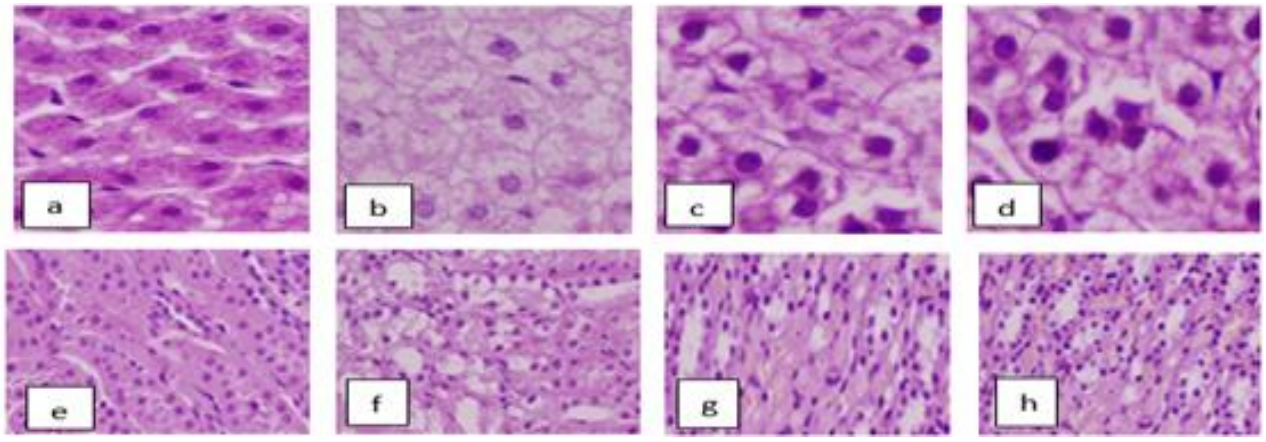

Fig. 1: Photomicrographs of rabbit liver (a-d) and kidney (e-h) microsections.
a- liver microsection from a normal control group showing normal histoarchitecture, b- liver microsection from paracetamol intoxicated control group showing necrosis and infiltration of inflammatory cells, c- liver microsection from PCM intoxicated group of rabbits treated with PI fruit methanol extract at a dose of 200 mg/kg showing repaired histoarchitecture with mild infiltration of inflammatory cells, d- liver microsection from PCM intoxicated group of rabbits treated with PI fruit methanol extract at a dose of 400 mg/kg showing repaired histoarchitecture with some infiltration of inflammatory cells, e- kidney microsection from a normal control group showing normal renal architecture, f- kidney microsection from paracetamol control group showing necrosis and epithelial desquamation, g- kidney microsection from PCM intoxicated group of rabbits treated with PI fruit methanol extract at a dose of 200 mg/kg showing improvement in renal architecture with some necrosis and epithelial desquamation, h- kidney microsection from PCM intoxicated group of rabbits treated with PI fruit methanol extract at a dose of 400 mg/kg showing improvement in renal architecture with some necrosis and epithelial desquamation.

respectively. The serum levels of urea and creatinine of PCM intoxicated rabbit group treated with 400mg/kg PI extract was 40.9% and 46.2% lower than the PCM control rabbit group, respectively. The serum urea and creatinine levels of PCM intoxicated rabbit group treated with 200mg mg/kg PI extract were not significantly different (P>0.05) from the serum urea and creatinine levels of PCM intoxicated rabbit group treated with 400 mg/kg PI extract.

During the present study, the creatinine clearance value of PCM control rabbit group was 62.1 % lower than the normal control rabbit group. The PCM rabbit groups treated with PI extract showed significantly higher creatinine clearance value when compared to the PCM control group. For example, the creatinine clearance values of PCM intoxicated rabbit groups treated with 200mg/kg PI extract and 400mg/kg PI extract were 40.44% and 74.2% higher than the PCM control rabbit group, respectively. The difference in creatinine clearance values between 200 mg/kg and 400mg/kg PI extract treated groups was significant (P<0.05).

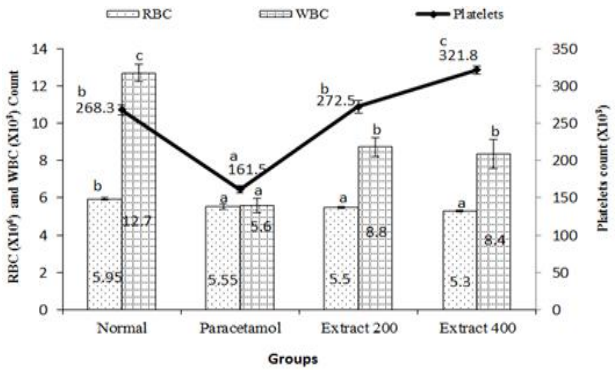

Fig. 2: Effect of PI fruit methanol extract on blood cells count of paracetamol-intoxicated male rabbits.
Means sharing no letter are significantly different at P<0.05 in Tukey test in One Way Anova.

Histopathological study of rabbit liver and kidney

The photomicrographs of rabbit liver microsections are shown in fig. 1 (a-d). Microsections from the livers of normal control rabbit group (fig. 1, a) showed normal histoarchitecture. Liver microsection from paracetamol control group (fig1, b) showed necrosis and infiltration of inflammatory cells. Liver microsections from PCM groups of rabbits treated with PI fruit methanol extract (fig. 1, c-d) showed improvement in liver histoarchitecture with mild infiltration of inflammatory cells. The photomicrographs of rabbit kidney microsections are also shown in fig. 1 (e-h). Microsections from the kidneys of normal control group (fig. 1, e) showed normal renal architecture. Microsection of kidney from paracetamol control group (fig. 1, f) showed necrosis and epithelial desquamation. Microsection from PCM groups of rabbits treated with PI fruit methanol extract (fig. 1, g-h) showed improvement in renal architecture with some necrosis and epithelial desquamation.

Effect of PI fruit methanol extract on blood cell count

The effect of PI extract on RBC, WBC and platelets count of rabbits was also studied (fig. 2). PCM control rabbit group showed insignificant decrease in RBC count as compared to normal control rabbit group (P>0.05). PI extract caused no improvement in the RBC count of PCM rabbit groups. During this study, PCM control rabbits showed significant decrease in WBC and platelets count when compared to normal control animals (P>0.05). PCM rabbits that received PI extract showed significant increase in WBC and platelets count when compared to PCM control rabbits (P> 0.05).

DISCUSSION

During the present study, the beneficial effect of *P. integerrima* fruit methanol extract on liver and kidney related blood biochemical parameters and haematological parameters was studied. Liver related parameters i.e., ALT, AST and ALP levels in serum were significantly higher in rabbits that received high dose of paracetamol. Microsections of liver sections from rabbits of PCM control group showed necrosis and infiltration of inflammatory cells (fig. 1, b). Sener *et al*. (2003) and Feola *et al*. (2017) also reported similar effect of high paracetamol dosing on the liver. Hepatic cells produce and possess the enzymes ALT, AST and ALP and higher levels of these enzymes are observed in the blood in case of damaged hepatic cells (Yoon *et al*., 2016; Marks *et al*., 2017; Saleh *et al*., 2018). Sharp increase in these enzymes in the blood is considered an important sign of damage in liver (Thapa and Walia, 2007). During the present study, high paracetamol dose also caused significant increase in serum urea and creatinine levels and significant decrease in creatinine clearance value. Microsection of kidneys from rabbits of paracetamol control group showed necrosis and epithelial desquamation (fig. 1, f). This shows the deterioration of renal architecture and function following the intraperitoneal administration of high PCM dose. Sener *et al*. (2003), Abraham (2005), Marks *et al*., 2017 and Saleh *et al*. (2018) also reported similar results after administration of high paracetamol dose. Excessive production of NAPQ1 due to paracetamol overdosing is responsible for renal toxicity (Whelton *et al*., 2003).

The PCM intoxicated rabbits treated with PI extract showed significant attenuation in the concentrations of ALT, AST and ALP in serum in a dose related fashion (table 1). Liver microsections from PCM groups of rabbits treated with PI fruit methanol extract showed improvement in liver histoarchitecture (fig. 1, c-d). Similarly, the PCM intoxicated rabbits treated with PI extract showed remarkable decrease in the levels of urea

and creatinine in serum, and elevation in urine output and creatinine clearance value in a dose related fashion (table 2). Microsections of kidneys from PCM groups of rabbits treated with PI fruit methanol extract showed improvement in renal architecture (fig. 1, g-h). These results demonstrate the nephroprotective effect of the PI extract. The observed hepatoprotective and nephroprotective effects of PI extract might be a consequence of the amelioration of oxidative stress and maintenance of the antioxidant capacity conferred by the extract. Plants contain high concentration of phenolic compounds, flavonoids and alkaloids they contain which possess antioxidant activity (Adeneye and Benebo, 2008). Plants phenolic compounds are very important because their hydroxyl groups possess radical scavenging ability and have the potential to prevent oxidative degradation of lipids (Kahkonen *et al*., 1999). Ahmad *et al*. (2006) reported the antioxidant activity of extracts of *P. integerrima* leaf and gall. Xanthine oxidase inhibitory activities of its leaf extract has also been reported (Ahmad *et al*., 2008). We have also explored the *in vitro* antioxidant activity of *P. integerrima* fruit methanol extract based on DPPH scavenging activity (Ilahi *et al*., 2013). The hepatoprotective activity of *P.* integerrima has also been reported by Khan *et al*. (2008). According to Ahmad *et al*. (2006), the gall of *P. integerrima* is helpful in reducing the high level of uric acid in the blood. Other medicinal plants have also been reported for their hepatoprotective and nephroprotective activities in PCM intoxicated animal models (Murad *et al*., 2015; Menyiy *et al*., 2018; Saleh *et al*., 2018).

During the present study, administration of high PCM dose resulted in insignificant decrease in RBC count when compared to normal rabbits (P>0.05) (fig. 2). Oyedeji *et al*. (2013) reported significant decrease in RBC count in rats after administration of high PCM dose. During the present study, high dose of PCM caused significant decrease in WBC count (fig. 2) which suggests that immune system was compromised. PCM intoxicated rabbits also showed significant (P<0.05) decrease in platelets count (fig. 2), which could be due to drug-induced immune thrombocytopenia (Bougie and Aster, 2001). During the present study, the oral administration of *P. integerrima* fruit methanol extract improved the red blood cell, white blood cell and platelets counts of paracetamol intoxicated rabbits (fig. 2). Such beneficial effects of other medicinal plants extracts on haematological parameters of PCM intoxicated animal models have been reported (Dougnon *et al*., 2011; Nwodo *et al*., 2013; Senthilkumar *et al*., 2014; Menyiy *et al*., 2018; Saleh *et al*., 2018).

CONCLUSION

From the findings of the present research, it was concluded that oral administration of *P. integerrima* fruit methanol extract is beneficial for the liver and kidney related biochemical parameters and blood cells count of paracetamol-intoxicated rabbits.

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