Indonesian Journal of Pharmacy

VOL 35 (3) 2024: 481–490 | RESEARCH ARTICLE

Attenuation of TNF-α and Iron Levels in Renal Hemosiderosis by *Phaleria macrocarpa* (Scheff.) Boerl Extract in a Rat Iron Overload Model

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Article Info

Submitted: 27-01-2023
Revised: 20-12-2023
Accepted: 15-01-2024

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ABSTRACT

Hereditary anemia known as thalassemia requires regular blood transfusions leading to an increase in total iron levels which can be toxic due to the formation of free radicals that damage cells and tissues. Oxidative stress and inflammation caused by excess iron can lead to renal disease progression. Mangiferin, an active constituent in *Phaleria macrocarpa* (Scheff.) Boerl (PM) fruit, has been shown to be an iron chelating agent whose complexes can facilitate excretion and reduce iron accumulation. This study aims to determine the effectiveness of an ethanolic extract of PM fruit as an iron-chelating agent in a rat model of iron overload. Thirty Sprague-Dawley rats were divided randomly into six groups: normal (N), iron overload (IO), iron overload treated with 462.5 mg/kg deferiprone (IO+D), iron overload treated with 50 mg/kg mangiferin (IO+M), and iron overload treated with 100 mg/kg PM extract (IO+PM100) and 200 mg/kg PM extract (IO+PM200). Iron overload was established by injection administered twice per week for 3 weeks and was continued for 8 weeks with treatment. Kidney iron levels were measured using AAS. Plasma urea and creatinine, and renal TNF-α levels were measured using assay kits. Kidney mangiferin levels were measured using HPLC. Mangiferin and PM extract cannot significantly reduce plasma urea and kidney iron levels and their effect on plasma creatinine levels was nonlinear. However, mangiferin and PM extract can reduce renal TNF-α levels significantly. Mangiferin and PM fruit extract have iron chelator ability and reduce inflammatory responses caused by iron overload.

Keywords: hemosiderosis, blood transfusions, iron metabolism, inflammation, iron chelator

INTRODUCTION

Thalassemias are inherited disorders of globin chain synthesis resulting in abnormal hemoglobin. More than 3% of people worldwide carry the thalassemia genetic disorder, with Southeast Asia having the highest incidence of up to 40%. Chronic anemia brought on by inefficient erythropoiesis, and intra and extramedullary hemolysis dominates the clinical picture of thalassemia (Laksmitawati *et al*., 2003). Currently, thalassemia patients are classified as transfusion-dependent (TD) or non-transfusion-dependent (NTD). While NTD patients frequently have anemia, they do not require blood transfusions for daily function or survival. However, TD thalassemia patients must receive regular blood transfusions throughout their lifetimes (Taher & Cappellini, 2021). Frequent transfusions can cause hemosiderosis, which describes a group of iron overload disorders causing an accumulation of hemosiderin and increased iron deposition in

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tissues, often without clinical signs (Udani *et al*., 2021).

Reactive oxygen species (ROS) and free radicals, which harm cellular and subcellular structures and result in metabolic dysfunction, are produced by metals like iron and some of their low molecular weight complexes. Disturbed iron metabolism can also produce free radicals (Laksmitawati *et al*., 2003). Excessive ROS production causes renal fibrosis and inflammation, and severe tissue damage, by accelerating lipid peroxidation, DNA damage, protein modification, and mitochondrial dysfunction (Jha *et al*., 2016). Increased oxidative stress has been shown to increase macrophage recruitment and MCP-1 levels. Furthermore, IL-6 and TNF-α levels were found to be strongly linked with the course of renal illness. IL-6 promotes mesangial cell proliferation, and TNF-α has been found to exert a positive feedback loop on ROS generation (Navarro *et al*., 2007).

Excess iron is known to be a risk factor for organ dysfunction and damage, resulting in diseases of various organs such as the liver, heart and kidney (Jha *et al*., 2016). Available free iron underlies iron toxicity because it accelerates the Fenton reaction, increasing ROS to levels that can saturate the antioxidant system. The human body can only manage iron to a certain extent by binding plasma iron to transferrin, which becomes highly saturated in individuals with iron overload. Plasma transferrin saturation results in labile plasma iron, or circulating non-transferrin-bound iron (NTBI), which may cause iron deposits in these organs (Sumneang *et al*., 2020). Apart from transferrin, the human body has no physiological mechanism to remove excess iron (Ho *et al*., 2013).

Iron overload is addressed with iron chelating agents, among which deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX) are currently approved by the FDA (Borgna-Pignatti & Marsella, 2015). However, the administration of iron chelating drugs should be monitored for possible adverse effects including allergic reactions, neutropenia, gastrointestinal disturbances, and kidney disorders (Rujito, 2019), and poor patient adherence is often reported with long-term use.

Phaleria macrocarpa (Scheff.) Boerl (PM) is a tropical, evergreen tree in the Thymelaeaceae family. Indigenous to Papua and Indonesia, its fruit has a long tradition of medicinal use to treat hypertension, diabetes, cancer, dysentery, rheumatism, and kidney disorders. The bark fruit contains alkaloids, saponins, and flavonoids, while the fruit contains alkaloids, tannins, flavonoids, mangiferin, phenols, saponins, lignans, essential oils, and sterols. Mangiferin is a glucosylxanthone that has been isolated from PM fruit with a reported extraction yield of 3.2% w/w (Hanif & Yuandani, 2020; Nurmaryam Aini *et al*., 2017). Studies indicate that mangiferin has broad pharmacological effects including antidiabetic, anti-HIV, anticancer, antioxidant, and immunomodulatory activities. The antioxidant activity of several polyphenolic compounds involved in the prevention of •OH radical formation and lipid peroxidation has been correlated with their activity as iron chelators (Andreu *et al*., 2005). Mangiferin has activity in lowering total plasma iron concentration in iron overload-induced rats (Estuningtyas, Wahyuni, *et al*., 2019). Excess iron can cause hemosiderosis, iron deposition in the kidneys, and damage to the renal system. The aim of this study was to determine the effect of PM extract on the kidneys, collecting and analyzing plasma and kidney tissue from rats in an iron overload model.

MATERIALS AND METHODS

Animals, materials and kits used in this study included Sprague-Dawley strain rats (Indonesia National Agency of Drug and Food Control, Indonesia), mangiferin standard (Sigma-Aldrich, US), mangiferin powder (Henan Senyuan Biological Technology, China), iron sucrose complex (Venover, US), ketamine injection, xylazine injection, ethanol, Urea FS kit (Diasys), Creatinine FS kit (Diasys), and TNF-α ELISA kit (Cusabio).

Study Design

The present study was designed as an experimental preclinical *in vivo* study using a model of iron overload in Sprague-Dawley rats, and was approved by the Health Research Ethics Committee of the Faculty of Medicine, University of Indonesia, and Cipto Mangunkusumo Hospital, No: KET-656/UN2.F1/ETIK/PPM.00.02/2020.

Methods

Thirty Sprague-Dawley rats were divided randomly into six groups: normal (N), iron overload (IO), iron overload treated with deferiprone at dosage 462.5 mg/kg BW (IO+D), iron overload treated with mangiferin at dosage 50 mg/kg BW (IO+M), iron overload treated with PM extract at a dosage of 100 mg/kg BW (IO+PM100), and iron overload treated with PM extract at dosage of 200

mg/kg BW (IO+PM200). To establish iron overload, iron sucrose was administered twice a week for 3 weeks, and administration was continued for 8 weeks with treatment. Blood samples were obtained from animals in each group by cardiac puncture after anesthesia. Kidney samples were also obtained from animals in each group and stored at -80°C. Kidney iron levels were measured using atomic absorption spectrophotometry (AAS) on a Shimadzu A-6300 (Japan). Plasma urea and creatinine levels were measured using commercial kits obtained from Diasys, and kidney TNF-α levels were determined using a kit from Cusabio.

Phaleria macrocarpa Extract Preparation

PM ethanol extract was prepared using a maceration method. To a maceration vessel was added powdered PM fruit and 70% ethanol (1:3), and the mixture was stored in cool, dark conditions for 3 days with occasional stirring. Filtration was carried out 3 times and the filtrate was concentrated on a rotary evaporator at 40-50 ºC until the extract thickened.

Kidney Fe Assay

Kidney tissue samples were digested using HNO_3, and then distilled water was added to the mixture. AAS (Atomic Absorption Spectrophotometry) measurements were made using deuterium as a light source (BGC-D2) at a wavelength of 450 nm.

Kidney Mangiferin Assay

Mangiferin was extracted from homogenized tissue samples (xx g) by adding acetosal (100 µM, xx mL) as an internal standard, 1 M HCl (xx mL), and acetonitrile (xx mL). The mixture was centrifuged at 1000 rpm for 10 min. The supernatant was separated and evaporated under nitrogen gas at 50°C. Methanol (200 µL) was added to the residue and the mixture was vortexed. The concentration of mangiferin was determined using High-Performance Liquid Chromatography (HPLC, Waters) with a Symetri C18 column (4,6 x 150 mm) and a 2998 Photodiode Array Detector (PDA, Waters) at a wavelength of 257 nm. Methanol and 0.5% formic acid (30:70) were used as the mobile phase applying a flow rate of 1 mL/min and a sample injection volume of 30 ul.

Plasma Urea and Creatinine Assay

Urea and creatinine kits were used according to the manufacturer's (Diasys) instructions. Reagent 1 and reagent 2 (4:1) were mixed. Samples (10 µL for urea testing and 50 µL for creatinine testing) were combined in a test tube with the reagent mixture. The mixture was homogenized and incubated for 1 min, and the absorbance was measured by spectrophotometry at a wavelength of 340 nm for urea and 492 nm for creatinine.

Kidney TNF-α Assay

Measurements were performed using a Rat ELISA kit from Cusabio. Homogenized samples were centrifuged for 5 min at 5000 g, and then stored overnight at -20 °C. 100 µL of each sample or standard was placed in a well, 100 µL Biotin-antibody and HRP conjugate were added with 60 min incubation for each addition, and the mixture was washed with PBS. Then, TMB substrate was added with 30 min incubation. A stop solution was added and the absorbance was measured at 450 nm.

RESULTS AND DISCUSSION

Mangiferin Levels in Kidney Organs

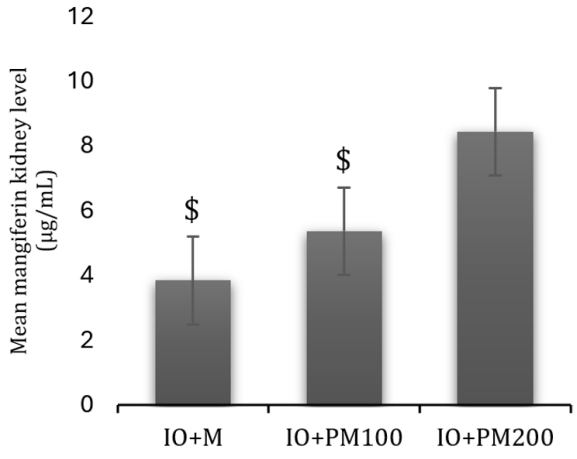
Mangiferin was detected in kidney organs of the iron overload groups treated with mangiferin and PM extract. The highest level was found at a PM dose of 200 mg/kg BW in the (IO+PM200) group, at levels significant compared to other treatment groups. The lowest mangiferin level was seen in the (IO+M) group treated with mangiferin at a dose of 50 mg/kg BW (Figure 1).

Iron Levels in Kidney Organs

The total iron levels in the kidneys of the iron overload group (IO) were significantly higher than that of the normal (N) and deferiprone-treated (IO+D) groups after 8 weeks of iron loading and treatment ($p < 0.05$). The IO group had 3.5 times the total iron levels of the N group. Both the IO+M and IO+PM200 groups showed a decrease in kidney iron levels, although the difference was not significant (Figure 2).

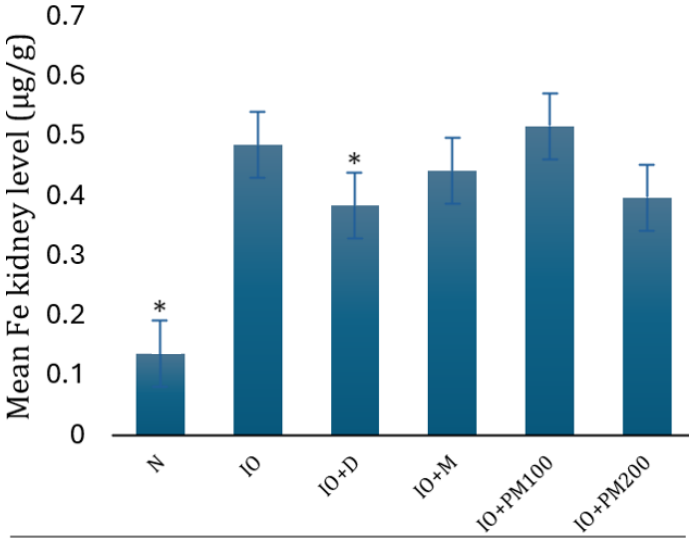
Urea Levels in Plasma

Plasma urea levels decreased in the treatment groups compared to the IO group after 8 weeks of iron loading and treatment. The IO group showed the highest level of plasma urea compared to other groups, 1.5 times that of the N group. The IO+M, IO+PM100 and IO+PM200 groups showed a nonsignificant reduction in plasma urea levels compared to rats in the IO group (Figure 3).



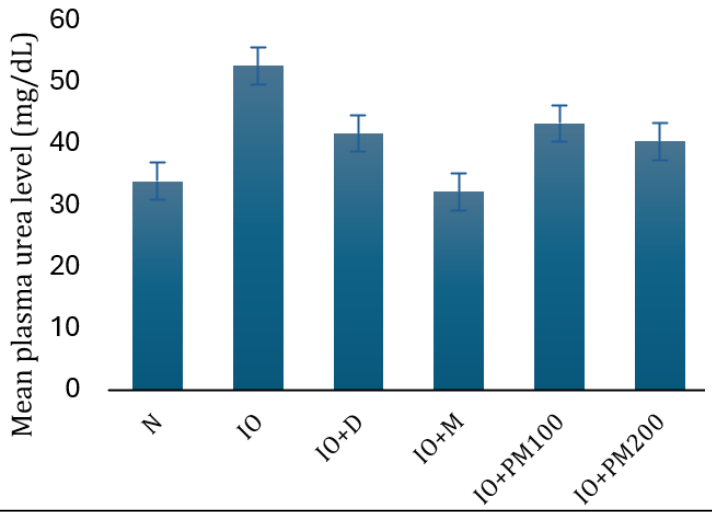
	IO+M	IO+PM100	IO+PM200
Mean	3.85	5.37	8.44
SEM	0.6100	0.6402	0.5112

Figure 1. Level of mangiferin in rat's kidney after 8 weeks of treatment
Description: three groups of animals contained 5 rats each.
IO+M = iron overload + oral mangiferin dose 50 mg/kg BW; IO+PM100 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 100 mg/kg BW; IO+PM200 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 200 mg/kg BW; $ = significant for IO+M and IO+PM100 compared with IO+PM200 ($p<0.05$).



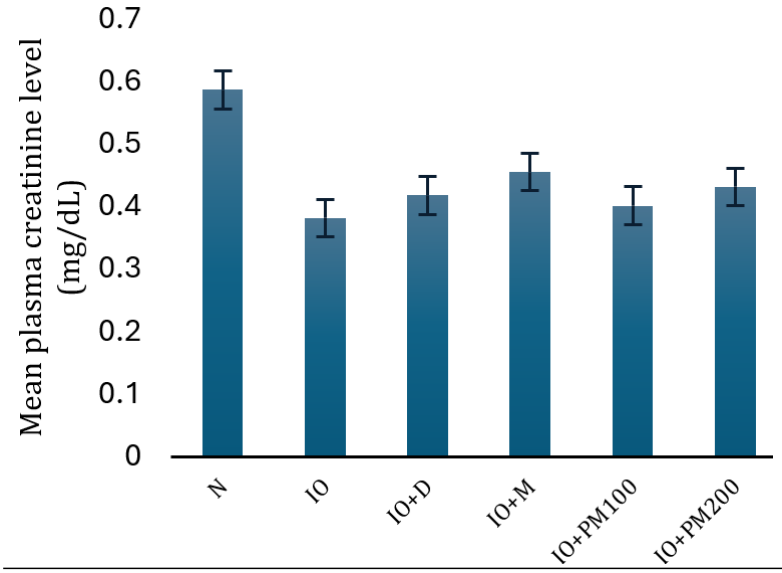
	N	IO	IO+D	IO+M	IO+PM100	IO+PM200
Mean	0.1371	0.4851	0.3838	0.4416	0.5168	0.3974
SEM	0.0671	0.0297	0.0297	0.0454	0.0477	0.0867

Figure 2. Level of iron in rat's kidney after 8 weeks of treatment
Description: six groups of animals contained 5 rats each.
N = normal; IO = iron overload; IO+D = iron overload + Deferiprone dose 462.5 mg/kg BW; IO+M = iron overload + oral mangiferin dose 50 mg/kg BW; IO+PM100 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 100 mg/kg BW; IO+PM200 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 200 mg/kg BW; * = significant for N and IO+D compared with IO ($p<0.05$)



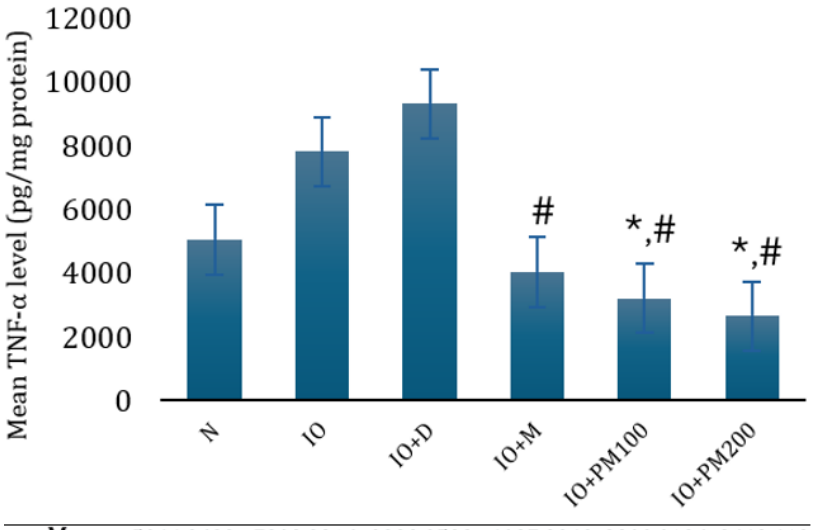
Mean	33.8428	52.6257	41.6124	32.1287	43.2517	40.3253
SEM	2.9089	11.879	3.4619	2.6191	6.6282	2.4429

Figure 3. Mean plasma urea level after 8 weeks of treatment
Description: six groups of animals contained 5 rats each.
N = normal; IO = iron overload; IO+D = iron overload + Deferiprone dose 462.5 mg/kg BW; IO+M = iron overload + oral mangiferin dose 50 mg/kg BW; IO+PM100 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 100 mg/kg BW; IO+PM200 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 200 mg/kg BW.



Mean	0.5868	0.3821	0.4178	0.4555	0.4016	0.432
SEM	0.0528	0.0418	0.0789	0.0169	0.0441	0.0296

Figure 4. Mean plasma creatinine level after 8 weeks of treatment
Description: six groups of animals contained 5 rats each.
N = normal; IO = iron overload; IO+D = iron overload + Deferiprone dose 462.5 mg/kg BW; IO+M = iron overload + oral mangiferin dose 50 mg/kg BW; IO+PM100 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 100 mg/kg BW; IO+PM200 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 200 mg/kg BW



	N	IO	IO+D	IO+M	IO+PM100	IO+PM200
Mean	5044.8688	7809.2916	9298.0598	4027.3948	3200.0196	2649.419
SEM	813.4774	1959.4826	1955.4992	652.9617	958.4681	162.4117

Figure 5. TNF-α levels in kidney organ after 8 weeks of treatment
Description: six groups of animals contained 5 rats each.
N = normal; IO = iron overload; IO+D = iron overload + Deferiprone dose 462.5 mg/kg BW; IO+M = iron overload + oral mangiferin dose 50 mg/kg BW; IO+PM100 = iron overload + ethanol extract of *Phaleria macrocarpa* (Scheff.) Boerl dose 100 mg/kg BW; IO+PM200 = iron overload + ethanol extract *of Phaleria macrocarpa* (Scheff.) Boerl dose 200 mg/kg BW; * = significant for IO+PM100 and IO+PM200 compared with IO ($p<0.05$); # = significant for IO+M, IO+PM100 and IO+PM200 compared with IO+D ($p<0.05$)

Creatinine Levels in Plasma

Plasma creatinine levels decreased in the treatment groups compared with the normal group after 8 weeks of iron loading and treatment, although the difference was not significant (Figure 4).

TNF-α Levels in Kidney Organs

After 8 weeks of iron loading and treatment, compared to the IO group, TNF-α levels decreased in all treatment groups except the deferiprone treatment (IO+D) group. The IO group had 1.5 times the total renal TNF-α level of the N group. Compared to the normal group, treatment with mangiferin (IO+M group) could lower TNF-α levels, though not significantly. However, compared to the IO and IO+D groups, PM extract treatment in the IO+PM100 and IO+PM200 groups was seen to significantly lower TNF-α levels ($p <0.05$) (Figure 5).

Phaleria macrocarpa (Scheff.) Boerl (PM) fruit extract contains the mangiferin constituent, with a reported extraction yield of 3.2% w/w (Nurmaryam Aini *et al*., 2017). A wide range of pharmacological properties have been reported for mangiferin, including antidiabetic, anti-HIV, anticancer, antioxidative, and immunomodulatory activities (Andreu *et al*., 2005). Furthermore, mangiferin was found to have efficacy in lowering total plasma iron concentrations in an excess iron model in rats (Estuningtyas, Wahyuni, *et al*., 2019). Mangiferin facilitates iron excretion in urine, preventing a rise in plasma levels (Estuningtyas, Setiabudy, *et al*., 2019). In this study, compared to the PM treatment groups, treatment with mangiferin showed lower levels of mangiferin in the kidneys. This observation correlates with the poor lipid and water solubility of mangiferin that leads to low transmembrane permeability and poor bioavailability (Guo *et al*., 2019). In contact with GI fluid, mangiferin can aggregate into particles that, because of their size, are absorbed more irregularly and with greater difficulty in the GI tract (Estuningtyas, Setiabudy, *et al*., 2019). Therefore, oral administration of mangiferin presents a number of difficulties, mostly because of its poor solubility, low bioavailability, hepatic first-pass metabolism, and rapid P-gp efflux (Khurana *et al*., 2017).

Meanwhile, PM extract active constituents such as quercetin and kaempferol are believed to operate as P-gp inhibitors. According to Limtrakul *et al*, flavonols, specifically quercetin and kaempferol at a concentration of 30 M, can dramatically decrease P-gp expression and function, causing P-gp activity to be inhibited (Limtrakul *et al*., 2005). As a result, there is a greater absorption of mangiferin into cells by virtue of other active constituents in the PM extract that inhibit the drug efflux transporter.

While iron is necessary for proper cellular physiology, excessive intestinal iron absorption, as observed in hemochromatosis, causes iron deposition in parenchymal cells of multiple organs, resulting in cellular toxicity, tissue damage, and organ fibrosis. Iron-generated oxyradicals and lipid membrane peroxidation cause cellular damage. The sequestration of free iron mitigates the transit and storage of mobilizable iron, as well as extracellular matrix remodeling and intracellular signaling events linked with inflammatory and fibrogenic cytokines (Ramm & Ruddell, 2005).

When kidney iron levels were measured, it was seen that the IO+M and IO+PM200 groups might lower the overall iron concentration compared to the IO group. This suggests that mangiferin contained in the PM extract can form mangiferin:Fe(III) complexes which are more readily eliminated from the body (Pardo-Andreu *et al*., 2006). The iron chelating ability of mangiferin is associated with its antioxidant activity, which includes scavenging superoxide radicals, and preventing the generation of •OH radicals and lipid peroxidation (Andreu *et al*., 2005). In addition, other active constituents in the PM extract such as quercetin are also capable of acting as iron chelators. The ability of quercetin to sequester intracellular iron into an iron-quercetin complex that can cross biological membranes has also been correlated with its activity as an antioxidant (Baccan *et al*., 2012).

In clinical practice, assessing plasma creatinine and urea concentrations using established biochemical procedures is a standard approach for evaluating kidney function (Kovalčíkova *et al*., 2018). Impairment of kidney function can be determined by abnormal estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR) values (Lin *et al*., 2023). Creatinine, a phosphocreatine metabolite, diffuses into the blood from many organs and is constantly excreted by the kidneys under physiological conditions. A reduced glomerular filtration rate results in an increase in plasma creatinine. The liver produces urea, which is also expelled by the kidneys. Increases in plasma urea and creatinine are signs of altered renal function (Kovalčíkova *et al*., 2018). According to the findings of this study, after induction of iron overload for a total of 11 weeks, there was a deterioration in renal function evinced by an increase in plasma urea levels. In contrast, creatinine levels did not reveal a reduction in renal function. Hence, the induced iron overload did not appear to cause significant harm to kidney function.

The IO group showed higher plasma urea levels than the other groups, 1.5 times that of the normal group after 11 weeks of iron loading. According to Ige *et al*., iron overload induction for 21 days caused an increase in plasma urea levels in Wistar rats (Ige *et al*., 2019). While plasma urea measurements in this study suggest a decreased ability of the kidney to excrete metabolic products, because plasma urea concentrations depend on hydration, protein intake, and liver function, they cannot accurately characterize the state of kidney injury (Wani & Pasha, 2021). Urea clearance is a measure of abnormalities in the glomerular filtration rate (Gowda *et al*., 2010). Plasma urea levels in the mangiferin and PM extract treatment groups tended to be reduced to levels that were nearly normal. This aligns with research utilizing an animal model of hyperuricemic nephropathy (HN) in which an increase in serum uric acid levels, a drop in serum blood urea nitrogen (BUN) levels, and a leveling of serum creatinine levels were all prevented by treatment with mangiferin (Li *et al*., 2020).

Regarding the creatinine measurements in this study, rats in the normal group without iron overload stimulus had higher mean plasma creatinine levels than those in the IO group. This is most likely caused by variations in muscle mass between groups. The breakdown of creatinine phosphate in muscles results in creatinine, which the body typically produces at a fairly steady rate depending on muscle mass (Gowda *et al*., 2010). Chromogen, excess kidney excretion, and a loss in muscle mass are further issues with interpreting serum creatinine levels. Chromogens are substances that can affect plasma creatinine readings and colorimetric reactions, increasing the value of yields by up to 20% (Rodrigo *et al*., 2002).

Regarding renal TNF-α levels, the IO group had 1.5 times that of the normal group, an increase in line with a similar iron overload model in Wistar

rats (Ige *et al*., 2019). After treatment with mangiferin and PM extract, TNF-α levels decreased significantly in comparison to the IO group. Depending on the dose, *in vitro* tests with mangiferin showed a dramatic suppression of PGE2 and NO generation, suppression of LPS-induced release of the proinflammatory cytokines TNF-α, IL-1, and IL-6, and increased expression of the anti-inflammatory cytokine IL-10 (Jeong *et al*., 2014). Mangiferin reduces inflammation by preventing activation of the JNK pathway and the NLRP3 inflammasome, a multi-protein complex involved in the NF-KB signaling pathway (Akter *et al*., 2022). According to Sundari *et al*, an aqueous extract of PM fruit can considerably lower TNF-α levels in a carbon tetrachloride-induced mouse model of liver fibrosis (Sundari *et al*., 2018). Acute kidney injury (AKI) processes are multifaceted, primarily involving inflammation, apoptosis, and oxidative stress. When normal physiological balance of oxidant and antioxidant biosynthesis is disrupted, oxidative stress is a significant contributing factor to AKI (Chen *et al*., 2019). Alkaloids, flavonoids, and polyphenols, which are known to have anti-inflammatory, antioxidant, anticancer, antidiabetic, antihypertensive, and hepatoprotective activities, are abundant in PM fruit extract.

CONCLUSION

In a rat iron overload model, the groups that received *Phaleria macrocarpa* (Scheff.) Boerl (PM) fruit extract showed renal mangiferin concentrations notably higher than that of the group treated with mangiferin. Kidney iron levels decreased, albeit not significantly, in the mangiferin and PM (200 mg/kg) groups demonstrating the ability of mangiferin to chelate iron and facilitate its excretion. Mangiferin and PM fruit extract did not significantly lower plasma urea levels, and their effect on plasma creatinine levels was not linearly correlated. In this study, although chronic kidney impairment had not yet developed, it was sufficient to demonstrate a decline in renal capacity to eliminate metabolic waste like urea. Compared to the model iron overload group, TNF-α levels were reduced in the groups treated with mangiferin and PM extract, demonstrating the capacity of mangiferin and PM to inhibit the inflammatory response and the generation of proinflammatory cytokines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The author is grateful for the grant funding to Penelitian Dasar Unggulan Perguruan Tinggi 2021 (PDUPT) No. NKB-097/UN2.RST/HKP.05.00/2021

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