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J.Biomed.Transl.Res
ISSN: 2503-2178

JOURNAL OF BIOMEDICINE AND TRANSLATIONAL RESEARCH

Available online at JBTR website: https://jbtr.fk.undip.ac.id

Original Research Article

Biochemical and Histopathological Effect of Combination Extract Ethanolic Turmeric (*Curcuma longa*) and Kalmegh (*Andrographis paniculata*) in Iron Overload Rat Model

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Article Info
History
Received: 10 Aug 2025
Accepted: 26 Jan 2026
Available: 30 Apr 2026

Abstract

Background: Thalassemia-β patients have erythrocyte damage that requires blood transfusion treatment. Repeated blood transfusions lead to iron overload in the body. The condition of iron overload will result in oxidative stress and organ damage that requires additional therapy

Objective: This study aimed to compare the effectiveness of turmeric and kalmegh extract doses against biochemical parameters and histopathological features of iron-induced liver *Rattus Norvegicus*.

Methods: This study used a True-experiment research design with a post-test-only control group. A treatment groups, normal, deferiprone drugs, turmeric and kalmegh extracts at doses of 100, 200, and 400 mg/kgBW. Malondialdehyde, catalase, superoxide dismutase, and ferritin are used as biochemical parameters and hepatic histopathology is documented.

Results: The results showed significant improvements in biochemical markers and liver histology in treated groups compared with the iron overload group.

Conclusion: The study also showed comparable effects to deferiprone.

Keywords: *Iron Overload; Kalmegh; Oxidative stress; Thalassemia; Turmeric.*
Permalink/ DOI: https://doi.org/10.14710/jbtr.v12i1.28945

INTRODUCTION

β-thalassemia with iron overload is a significant clinical problem β-thalassemia with iron overload is a significant clinical problem. Thalassemia-β patients with iron overload conditions are reported in some countries such as Thailand, with a total of 405 individuals, the majority of whom were male (46.7%) with 21 individuals (5.2%) having iron overload in the heart, and 230 individuals (56.8%) in the liver.[1] Thalassemia-β patients with iron overload were also reported in Germany as much as 17.74% or about 447 individuals. In addition, reports in the Southeast Asia region (Malaysia, Hong Kong, Indonesia, Taiwan, and Singapore) have the highest prevalence of 53% of thalassemia-β patients in iron overload conditions.[2]

Iron overload in β-thalassemia results from increased intestinal iron absorption.[3,4] This is due to failure in the formation of β-globin expression.[5,6] The lack of globin-β expression is caused by mutations such as CD60 (GTG > GAG) which makes hemoglobin easily broken and requires regular blood transfusions.[7–9] However, Regular blood transfusions further contribute to iron overload.[10,11]

The liver is the main organ that plays a role in the storage and distribution of iron.[12] Iron overload induces oxidative stress in the liver, leading to cellular and tissue damage resulting in an imbalance of free radicals and endogenous antioxidant activity in the body.[13–15] Thalassemia-β patients require supportive therapy to increase antioxidant activity and lower iron levels in the body.[16,17] Turmeric (*Curcuma longa*) and Kalmegh (*Andrographis paniculata*) are plants that can be developed into supportive therapy because it has antioxidant compounds.[18,19]

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Kalmegh is a diterpenoid contain able to reduce oxidative stress levels and can significantly increase the total level of antioxidant enzymes.[19] Kalmegh can also be a therapy in repairing liver tissue damage assessed based on the improvement of periportal histological scores and briding necrosis.[20,21] This also applies to turmeric, Turmeric contains flavonoid compounds that are able to significantly reduce stress oxidative levels and be able to activate iron regulatory proteins (IRPs) so that it is useful for binding iron.[22] In addition, turmeric is able to significantly increase antioxidant enzymes as measured by improvements in the antioxidant enzyme index and histopathological liver lesion conditions.[23]

Studies on the use of these two extracts are still very limited, on average only using a single dose, and application to an iron overload model has never been carried out. So, this study aims to determine the differences in biochemical and histopathological effects of *Rattus norvegicus* liver-induced iron sucrose after turmeric and kalmegh extract. In addition, this study was conducted to see the effectiveness of turmeric and kalmegh extracts as supportive therapy in rat models of iron overload or iron overload conditions of thalassemia-β patients.

MATERIALS AND METHODS

Study Design

This study used a True Experimental design with a post-test-only control group design. The study animal used was a male *Rattus norvegicus Wistar* strain with a body weight of 250-300g, age ranging from 8 weeks obtained from UD. Wistar Bantul as many as 30 rats (Certificate No: 62/WS/14/03/2023). Animals were placed in the cage of the experimental animal laboratory, Faculty of Medicine, Universitas Jenderal Soedirman. Rats get fed and drink ad libitum. The induction material for making iron overload models is Iron sucrose (CAS: 6047-67-4) obtained from Solarbio Life Sciences. The rats used were divided into six groups: Normal Group (GN) (n=5), Group induced iron overload (GIO) (n=5), Group of control with Deferiprone (GC) (n=5), and three groups with iron overload were given turmeric and kalmegh extracts at doses of 100mg/KgBW (E100) (n=5), 200mg/KgBW (E200) (n=5), and 400mg/KgBW (E400) (n=5).

Iron overload was induced using iron sucrose at 15 mg/kgBW, administered twice weekly for four weeks (a total of 8 times). Turmeric and kalmegh extracts were administered orally, daily with a volume of 2-3mL depending on the body weight of the rats. A 3 mL plasma blood sample was taken in the periorbital vein for measurement of biochemical parameters such as malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and ferritin. Iron overload group (GIO) blood was drawn at week 4, while the other group was drawn after treatment at week 8.

Extract Preparation

Turmeric and kalmegh simplicia were purchased from IndoPlant Lansida Group Herbal Technology Yogyakarta Licensed by the Ministry of Health of the Republic of Indonesia in powder form. The powder weight of each plant is 1000 grams. Extraction was carried out by maceration with occasional stirring to accelerate the release of active substances into the solvent. The solvent used was 1 liter of 96% ethanol for each powder, soaked for 5 days. The resulting of yield percentage is 5.38% and after macerated is then evaporated with a rotary evaporator at a temperature of 50 °C.

Biochemical Analysis

The biochemical activity of MDA, CAT enzyme, and SOD enzyme was measured by UV-Vis spectrophotometry method according to standard kit measurement from Solarbio Life Sciences. MDA activity was measured from a 200 µL blood plasma sample. Reagents and buffers were prepared, mixed, and incubated at 100 °C for 60 minutes. Then 10000xg centrifugation was carried out for 10 minutes at room temperature which was then read at waves of 532 nm and 600 nm. MDA activity expressed in nmol/mL. On the activity of the CAT enzyme using 35µL from a blood plasma sample with added 1 mL of CAT Working reagent mixed for 5 seconds then read at a 240 nm wave. The activity of the CAT enzyme is expressed in U/mL.

SOD enzyme activity is measured using xanthine and xanthine oxidase (XOD) to form red formazan, using a blood plasma sample of 180 µL divided into two samples, namely control and test. The sample was centrifuged for 10 minutes at a speed of 400 rpm. Measurements of SOD enzyme activity were read at 560 nm waves and expressed in U/mL. Ferritin measurement using the ELISA Sandwich method according to Solarbio Life Sciences standard guidelines. The blood plasma sample used was 100 µL and incubated for 90 minutes. The wavelength used is 450 nm with Ferritin activity expressed in ng/mL.

Histopathological Preparation and Examination

The liver organ is cut into sizes of 1x1x1 cm then fixated in a 10% formalin solution for 24 hours, then embedded in paraffin. The organ is cut to a thickness of 4-5µm then stained with hematoxylin and eosin (H&E). Then examined and photographed using a microscope with a magnification of 400x. Histopathological examination at Anatomy and Pathological Laboratory Waskitha Yogyakarta. The degree of histopathological degeneration is defined as 0=normal, 1=focal (damage in one area), 2=multifocal (damage in several areas), 3= diffuse (severe damage or in all areas).[24]

Statistics

The measurement data was analyzed using SPSS V.26.0. The One-Way ANOVA Test is used for biochemical parameters which was followed by a Post Hoc LSD test to determine the differences between groups, while the Mann-Whitney Test is used for histopathological parameters. A value of $p<0.05$ is considered statistically significant.

Ethics

Research ethics is published by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Jenderal Soedirman with an Ethics approval number 072/KEPK/PE/V/2023.

RESULTS

Comparative Effect of turmeric and kalmegh extracts with deferiprone on biochemical parameters

In this study, it was found that MDA levels of Group-induced iron overload (GIO) had very high levels of MDA compared to the normal group (GN), and the difference between these groups was statistically significant ($p<0.05$) described in Table 1.

The results of this study were found that the combined effect of turmeric and kalmegh extract (EI, EII, EIII) showed a difference with the drug deferiprone (GC). The data shown in Table 1 showed that the combination extract group of turmeric and kalmegh at a dose of 400mg/KgBW had a malondialdehyde value or oxidative stress level of 2.09±0.58 nmol/mL, closest to the drug deferiprone of 2.07±0.71. The levels of CAT and SOD enzymes were also described that the dose of 400mg/KgBW had approximately the same values, namely 79.96±3.96 U/mL (EIII) and 36.76±3.99 U/mL (EIII), with the deferiprone group. Likewise, the ferritin level where the value of ferritin as a supporter of iron accumulation in the body is lowest at a dose of 400mg/KgBW 141.84±23.75 ng/mL in contrast to the dose of deferiprone which is at 210.71±31.89 ng/mL. The dose of 400mg/KgBW showed the most effective dose in lowering MDA and ferritin levels and improving the activity of the enzyme CAT, SOD. To facilitate the visualization of comparisons between test groups, it can be seen in Figure 1.

Effects of turmeric and kalmegh extract on histopathological parameters

Histopathology of rat liver in the normal group is indicated by the condition that hepatocyte cells appear normal and clear (Figure 2.A). In difference to the group given iron induction (Figure 2.B) Indicates necrosis, cytoplasm appears to be damaged and boundaries between cells are not visible. Based on the Kruskall-Walis statistical analysis, the results showed that the variation in the combination dose given orally had a

Table 1. Effects of turmeric and kalmegh extracts on biochemical levels in iron overload model rats (n = 30)

Group	MDA (nmol/mL) Mean±SD	CAT (U/mL) Mean±SD	SOD (U/mL) Mean±SD	Feritin (ng/mL) Mean±SD
GN	2.38±0.80$^{\#a}$	80.16±12.28$^{\#ab}$	33.5±6.35	168.43±28.22$^{\#a}$
GIO	13.22±1.24^{*abcd}	54.46±7.46^{*abcd}	18.15±3.07^{*abcd}	262.13±25.34^{*bcd}
GC	2.07±0.71$^{\#a}$	76.54±3.63^{ad}	33.61±5.21	210.71±31.89$^{\#d}$
EI	4.11±0.49$^{*\#abcd}$	66.92±1.40^{*bd}	27.59±3.65^{d}	238.09±41.92^{*cd}
EII	3.05±0.49$^{\#a}$	68.74±7.10^{bd}	32.07±4.42$^{\#}$	171.93±46.62$^{\#a}$
EIII	2.09±0.58^{bc}	79.96±3.96^{ac}	36.76±3.99$^{\#a}$	141.84±23.75$^{\#ab}$

Description: GN: Normal Group, GIO: Group Induced-Iron Overload, GC: Group of Control Deferiprone, EI: Extract 100mg/KgBW, EII: Extract 200mg/KgBW, EIII: Extract 400mg/KgBW. MDA: Malondialdehyde, CAT: Catalase, SOD: Superoxide dismutase. *= p<0,05 vs GN, $^{\#}$= p<0,05 vs GIO, ^{a}= p<0,05 vs EI, ^{b}=p<0,05 vs GC, ^{c}=p<0,05 vs EII, ^{d}=p<0,05 vs EIII. Analysis One Way ANOVA Posthoc LSD.

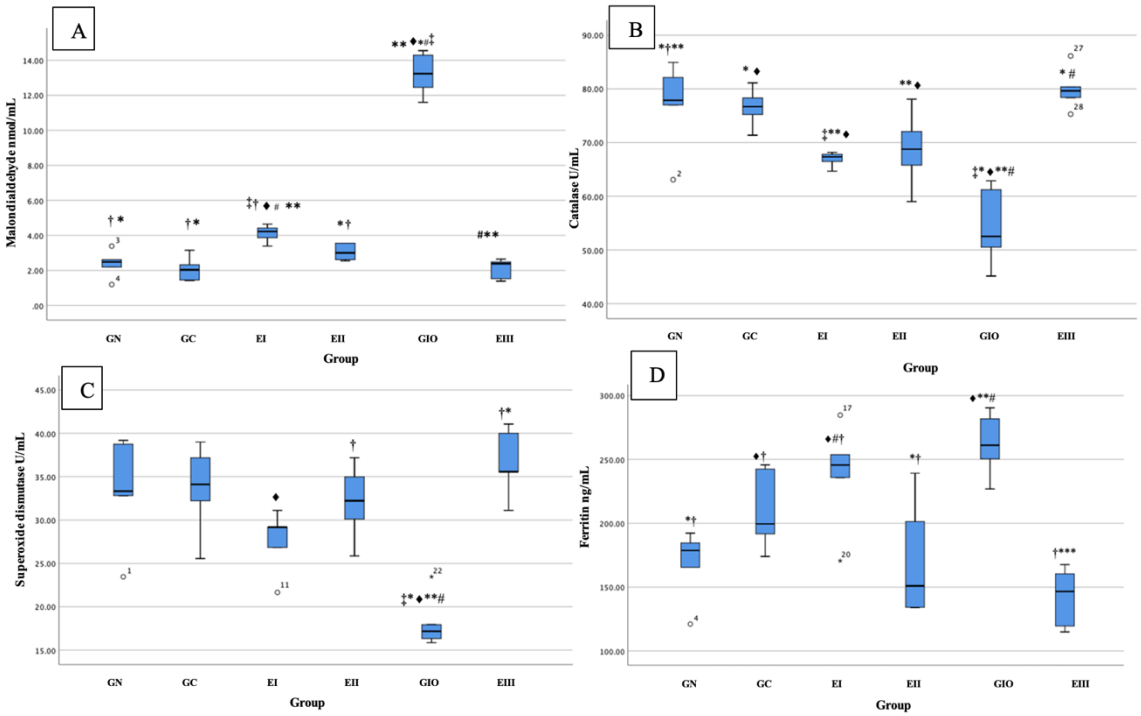

Figure 1. Boxplot showing comparison of biochemical levels. A) Malondialdehyde B) Catalase C) Superoxide dismutase D) Ferritin. GN: Normal Group, GIO: Group Induced-Iron Overload, GC: Group of Control Deferiprone, E: Extract 100mg/KgBW, EII: Extract 200mg/KgBW, EIII: Extract 400mg/KgBW. ‡= vs (*G*N), †= vs (GIO), **=vs (GC), *=vs (EI), #= vs (*E*II), ♦=vs (*E*III).

Table 2. Score Histopathology

Group	Degeneration Score				Necrosis Score				Congestion Score				Infiltration Score			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
GN$^{\#abc}$	5	-	-	-	5	-	-	-	5	-	-	-	5	-	-	-
GIO^{*ad}	2	3	-	-	-	5	-	-	4	-	1	-	2	3	-	-
GC^{acd}	4	1	-	-	1	4	-	-	5	-	-	-	2	-	-	3
EI$^{*\#bcd}$	2	3	-	-	2	3	-	-	-	5	-	-	1	-	-	4
EII$^{*\#abd}$	-	-	-	5	2	3	-	-		5	-	-	-	-	-	5
EIII$^{*\#c}$	5	-	-	-	4	1	-	-	2	3	-	-	-	-	-	5

Description: *= p<0,05 vs GN, $^{\#}$= p<0,05 vs GIO, ^{a}= p<0,05 vs EI, ^{b}=p<0,05 vs GC, ^{c}=p<0,05 vs EII, ^{d}=p<0,05 vs EIII. Analysis Kruskall-Walis Posthoc Mann-Whitney.

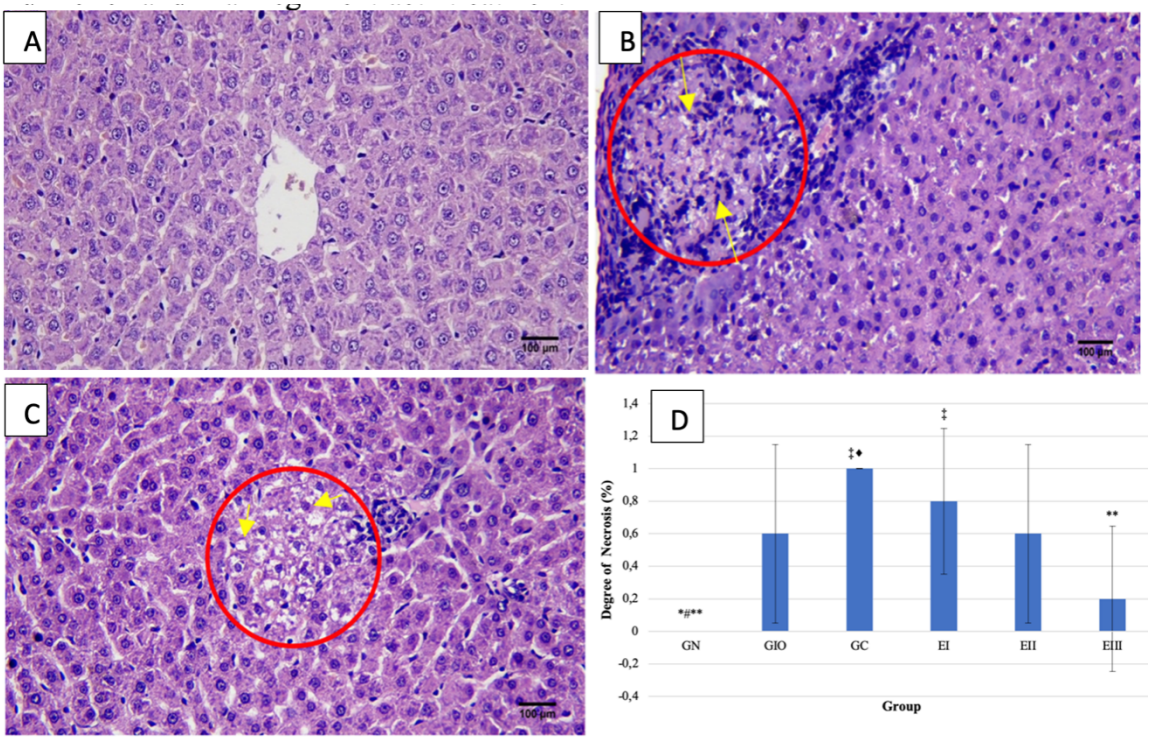

Figure 2. Microscopic Test Differences in iron-induced rat liver histopathology. (A) normal hepatocytes, (B) necrosis, (C) hydropic degeneration, (D) Differences in the degree of necrosis after administration of the extract. GN: Normal Group, GIO: Group Induced-Iron Overload, GC: Group of Control Deferiprone, E: Extract 100mg/KgBW, EII: Extract 200mg/KgBW, EIII: Extract 400mg/KgBW. ‡= vs (GN), †= vs (GIO), **=vs (GC), *=vs (EI), #= vs (EII), ♦=vs (EIII)

significant effect on the degree of necrosis (p = 0.019), degeneration (p = 0.001), congestion (p = 0.001), and infiltration (p = 0.001). This was then continued with the Mann-Whitney difference test (Table 2). Turmeric and kalmegh extract treatment showed improvement of hepatocyte cells in Figure 2.C, although hydropic degeneration still occurred where the cytoplasm appears pale. Administration of 400mg/KgBW extract show minimal lesions compare to deferiprone drug administration shown in Figure 2.D.

DISCUSSION

In the results of this study, it was found that the induction group (GIO) obtained high stress oxidative levels characterized by high malondialdehyde parameters of 13.22±1,24 nmol/mL. The impact of increased MDA is a decrease in the activity of endogenous antioxidant enzymes SOD and CAT due to failure to respond to the cellular defense system against oxidative stress. The results show that there is a decrease in SOD levels from 33.5±6.35 U/mL (GN) become 18.15±3,07 U/mL (GIO), while the CAT level of 80,16±12,28 U/mL become 54.46±7,46 U/mL (GIO).

The antioxidant enzymes SOD and CAT are found in the liver, but the liver also plays an important role in regulating iron.[25] Iron in the body is stored in the form of ferritin protein, so an increase in iron can be noticed by an increase in ferritin levels.[26] Referring to the results of this study, it was found that ferritin levels increased in the induction group by 262,13±25,34ng/mL when compared to normal groups by 168,43±28,22 ng/mL. The data presented in Table 1 showed that several treatment groups that had been given turmeric and kalmegh extracts showed an improvement in both antioxidant enzyme activity and a decrease in ferritin levels. This is because turmeric has flavonoid compounds and kalmegh has a diterpenoid that can inhibit or bind iron.[27] Flavonoids and diterpenoids are able to neutralize harmful free radicals because they have phenolic and methoxy groups so that they are able to induce

endogenous antioxidant defense mechanisms by minimizing NfkB activity.[28] Turmeric and kalmegh also have phenolic groups (OH) that can be scavengers for free radicals.[29,30]

The results of this study are the same as previous research which explained that turmeric extract was able to reduce oxidative stress parameters from the initial value of 2.920± 0.529 ng/mL become 1.403±0.444 ng/mL.[31] Likewise, kalmegh extract was able to improve the activity of the antioxidant enzyme SOD from 2.2 U/mL to 3.6 U / mL, while CAT from 5.1 U/mL to 8.1 U/mL.[32]

Based on histopathological testing, it was found that the degree of damage, hydropic degeneration, and necrosis were found (Figure 2.B, 2.C). The findings in this study showed that the dose of the drug had a high level of necrosis with 1% decree of necrosis (Figure 2.D) due to the occurrence of excessive reactive metabolites.[33]However, the study dose of 400 mg/KgBW had a necrosis damage rate of 0.2%.

CONCLUSION

It can be concluded that turmeric and kalmegh extracts have compounds that are contain bioactive compounds with antioxidant properties for the reduction of iron overload. Turmeric and kalmegh extract can lower malondialdehyde levels depending on the dose of the extract, with a dose of 400mg/KgBW having the best reduction. The decreasing effect of malondialdehyde synergizes with low ferritin values. In antioxidant enzyme enhancement the dose of 400mg/KgBW has the best improvement. The literature on kalmegh as an iron chelator is very limited, it would be good to test the ferrous ion chelating of a single extract in-vitro.

ACKNOWLEDGMENTS

Thanks to BPPT (Balai Pembiayaan Pendidikan Tinggi) Beasiswa Pendidikan Indonesia, Kementerian Pendidikan Kebudayaan Riset dan Teknologi, Lembaga Pengelola Dana Pendidikan (LPDP) The Republic of Indonesia has funding this research process, and Thanks to Direktorat Jenderal Riset dan Pengembangan Kementerian Pendidikan Tinggi Sains dan Teknologi for publication funding.

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