ORIGINAL RESEARCH REPORT

Potential of Red Okra (*Abelmoschus esculentus* (L.) Moench) Ethanol Extract to Protect Against 7, 12-Dimethylbenz[a]anthracene-Induced Damage in Rat's (*Rattus norvegicus*) Liver

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ABSTRACT

Background: Okra (*Abelmonchus esculentus* (L.) Moench) is a plant that has potential for humans and health because it contains high antioxidants such as polyphenols. **Objective**: This study aimed to analyze the red okra pods' ethanol extract (ROE) antioxidant potential to protect rat (*Rattus norvegicus* L.) liver against damage by induction of 7, 12-dimethyl-benz[a]anthracene (DMBA). **Material and Method**: The material used was various doses of red okra pods with ethanol as solvent. Twenty-five female rats (4 weeks, body weight 140—150 g) were divided into five groups: negative control (receiving a single dose of 0.5 mL corn oil as DMBA solvent), positive control (receiving a single dose induction of DMBA 80 mg/kg BW dissolved in 0.5 mL corn oil), and treatment groups 1, 2, 3 (receiving a single dose of DMBA 80 mg/kg BW dissolved in 0.5 mL corn oil and ROE of 50, 100, and 200 mg/kg BW, respectively, daily for 35 days). On day 36, the livers were removed and prepared for histopathological observation using hematoxylin-eosin staining. The method of histopathological score was determined using an ordinal score. **Result**: The data were analyzed statistically ($p>0.05$). All ROE doses showed a significant decrease in the histopathological score of rats' liver damage caused by DMBA-induced. The liver protection with ROE reduced the percentages of reversible and irreversible cellular damage from 51.8% to 35%, 27.3%, and 18.9%, respectively. **Conclusion**: The red okra pod ethanol extract can protect against DMBA-induced liver damage in rats.

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Highlights

1. Red okra pods have a potential antioxidant to protect against DMBA-induced damage in a rat's liver.
2. The liver protection with ROE decreased reversible and irreversible cellular damage from 51.8% to 35%, 27.3%, and 18.9%.

BACKGROUND

7, 12-dimethylbenz[a]anthracene (DMBA), one of the polycyclic aromatic hydrocarbons (PAHs), is a persistent organic pollutant found in the environment. Humans can be exposed to these pollutants daily because they are mostly generated by the incomplete combustion of organic materials such as gasoline, coal, cigarettes, oil, industry, and transportation (Song, et al., 2017; Selamoglu, 2018; Abdelmeguid, et al., 2021). This compound is genotoxic and tumorigenic, so its bioaccumulation can be a potential carcinogen. All chemical carcinogens or their derivatives have reactive electrons that trigger the production of free radicals and can affect electron-rich cells such as nucleophiles, especially deoxyribonucleic acid (DNA), as it has one or more covalent bonds (Anwar, 2013; Hosny, et al., 2021; Plante, 2021; Yustisia, et al., 2022). Several studies have proven that DMBA can cause damage and tumor development in experimental animals, including the livers of the animals (Hosny, et al., 2021; Plante, 2021). The liver is the main organ responsible for all metabolic homeostatic activities, food and drug metabolism, and detoxification of both endogenous and exogenous substances (Kalra, et al., 2023). Under physiological conditions, liver parenchyma will produce endogenous antioxidants and oxidant reactive oxygen species (ROS) as a manifestation of cellular metabolic results. However, under pathological conditions such as exposure to DMBA, liver parenchyma will produce excess ROS, and an imbalance between endogenous antioxidants and oxidants can trigger oxidative stress. This situation causes liver cells to lose normal function and leads to microscopic structural damage (Li, et al., 2015; Ahmad, et al., 2018; Tan, et al., 2018; Ali, et al., 2020).

To overcome this, there is a growing interest in utilizing the rich antioxidants of plants as a cost-effective and accessible solution to reduce those side effects (Elkhalifa, et al., 2021). Plants have been utilized by about two-thirds of the world's population (7.8 billion) as medicinal materials for disease management because they contain natural antioxidant bioactive components, such as polyphenols, which capture, reduce, and inhibit the production of ROS oxidants. One of the plants with high antioxidants is okra (*Abelmoschus esculentus* (L.)) (Mani, et al., 2018; Elkhalifa, et al., 2021). The antioxidant content in okra plants was found to have the second highest total polyphenols contain (TPC) value among 15 different vegetable species that have been identified such as lemon, spinach, onion bulbs and leaves, zucchini, garlic, cabbage, and others (Fabianová, et al., 2022). The okra plant, nicknamed Lady's Fingers, belongs to the Malvaceae family of vegetables. Okra plants grow in the tropics, subtropics, and areas with warm temperatures. The okra plant is believed to have originated in Ethiopia and then spread to the Middle East and North Africa. Today, okra is a popular plant cultivated widely and globally. Some populations worldwide, especially in low-income countries, consume and make okra plants as a local staple food because of the high nutritional content that can be obtained at an affordable price (Elkhalifa, et al., 2021). In Indonesia, okra plants can be found in traditional markets (Wahyuningsih, et al., 2020). The okra varieties cultivated in Indonesia are red and green (Syam, et al., 2019). The part of the okra plant usually consumed is the elongated capsule-shaped pods (Elkhalifa, et al., 2021). Okra pods can be processed by frying, boiling, and extracting (Gemede, 2015). Okra pods are categorized as powerful antioxidants in terms of 2, 2-diphenyl-picrylhydrazyl (DPPH) antioxidant activity (Wahyuningsih, et al., 2020). The potent antioxidant comes from the high content of phenolics and flavonoids in okra pod extract, especially red okra pods, which has been reported as 45.083 ± 0.084 mg TAE/5g sample and 14.245 ± 0.078 mg QE/5g sample (Syam, et al., 2019). Thus, the antioxidant content of red okra pods was studied to see its ability to help maintain the balance of antioxidants and oxidants to protect against DMBA-induced damage in rat (*Rattus norvegicus* L.) livers.

OBJECTIVE

This study aimed to analyze the red okra pods' ethanol extract (ROE) antioxidant potential to protect rat (*Rattus norvegicus* L.) liver against damage by induction of 7, 12-dimethyl-benz[a]anthracene (DMBA).

MATERIAL AND METHOD

Study period and locations

This study used a randomized post-test control group design conducted in July—November 2022 at the Animal Laboratory, Histology Laboratory, Molecular Biology Laboratory, Faculty of Science and Technology, Universitas Airlangga, and at the Histology Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

Chemicals

Red okra pods were extracted using ethanol pro-analysis (96%) from Merck Millipore (Cat. No. 159010, Darmstadt, Germany). The other chemicals for histology preparations were hematoxylin (Cat. No. 115938), eosin (Cat. No. 15935), neutral-buffered formalin 10% (Cat. No. 100496), paraffin pastilles (Cat. No. 100496), ethanol 70%, ethanol 80%, ethanol 96%, ethanol 100% (Cat. No. 100983), xylene (Cat. No. 108297), and entellan mounting medium (Cat. No. 107961) obtained also from Merck Millipore (Darmstadt, Germany).

Plant preparation

This study collected red okra pods from a local Lowokwaru, Malang, Indonesia farm. Red okra pods were cleaned and air-dried with no contact and direct sunlight. Those dried red okra pods were pulverized in a mechanical grinder until they became powder. About 500g of dried red okra powder was extracted using 96% ethanol solvent (thrice, 24hours each), and the solvents were collected. Those red okra solvents were evaporated until freeze-dried using a rotary evaporator, and then the extract could be obtained.

Animals

Twenty-five female rats (4 weeks, 140—150 g) were obtained by the Animal Laboratory, Faculty of Veterinary, Universitas Airlangga, Indonesia. The cages for maintaining the animals were made of plastic with a lid made of woven wire cages at 20°C, with a 12°h light/12°h dark cycle. The animals were fed and watered ad libitum. The animals were acclimatized for 14 days before the experiment.

Administration of DMBA and ROE

The five treatment groups (K-, K+, P1, P2, and P3) contained randomly 5 rats per groups. Group 1 (K-) was administered with a single corn oil dose of 0.5 mL. Group 2 (K+) received a DMBA induction (a dose of 80 mg/kg BW dissolved in 0.5 mL corn oil). Groups 3-5 (P1-P3) received a DMBA induction (a single dose of 80 mg/kg BW dissolved in 0.5 mL corn oil) and were administered 50, 100, and 200 mg/kg BW of ROE for 35 days, respectively. The DMBA was administered intraperitoneally, while the ROE was administered orally.

Organ collection

The liver organs were collected using the ketamine-xylazine injection technique. The obtained samples from the sacrificed animals were washed using normal saline and fixed in neutral-buffered formalin (10%) for histological observation.

Histopathological study

The liver tissue samples were cut and fixed in neutral buffered formalin for 48 hours. The samples were processed manually, dehydrated in 70%, 80%, 96%, and 100% ethanol, and embedded in a paraffin block. Each section was cut at a thickness of 4 µm using a rotary microtome. Each section was stained by hematoxylin and eosin (H&E). The slides were examined under a light microscope (CX31

OLYMPUS with OLYMPUS U-TV0.5XC-3) (Tokyo, Japan) at 40x magnification. The central veins were observed for cellular structural changes, especially reversible and irreversible damage, and then scores were given based on the liver damage. The method of histopathological score was determined using an ordinal score.

Table 1. The histopathological scoring of the liver. Modified from Nurhidayat, et al., (2022).

Cellular damage	Score
No cellular damages	0
Reversible damages with necrosis in tissue less than 15%	1
Reversible damages with necrosis in tissue between 15—40%	2
Reversible damages with necrosis in tissue between 41—70%	3
Reversible damages with necrosis in tissue more than 70%	4

Statistical analysis

The statistical data analysis was performed using the descriptive method of one-way analysis of variance (one-way ANOVA) and followed by Duncan's post hoc test. All analyses were processed using the Statistical Package for the Social Sciences 26.00 for macOS (SPSS Inc., Chicago, IL, USA). The mean ± standard deviation (SD) was reported as the result of five replications (p>0.05).

RESULT

The histopathological score was applied to evaluate the ROE antioxidant potential to protect against DMBA-induced damage in the rat's liver. The measurement results were obtained by observing the cellular structural changes in the central veins, especially reversible and irreversible damage. The results of analyzing the histopathological scoring of the ROE to protect against DMBA-induced damage in rats' livers can be seen in Table 2 and Figure 1.

Table 2. Histopathological scoring of the ROE to protect against DMBA-induced damage in rats' liver.

Treatment groups	Mean ± SD	Kolmogorov-Smirnov	Levene	One-way ANOVA
K-	2.00 ± 0.14			
K+	2.96 ± 0.22			
P1	2.00 ± 0.14	0.057	0.838	0.000*
P2	1,56 ± 0.17			
P3	1.08 ± 0.11			

*Significantly different (p<0.05)

The obtained data were statistically analyzed descriptively to show each group's mean and SD in Table 2. The first test performed was the Kolmogorov-Smirnov (p>0.05). The value was significant (p=0.057) and categorized as normal data. The second test performed was the Levene (p>0.05). The value was significant (p=0,838) and classified as homogenous data. Because the data was normal and homogenous, the third test performed was a one-way ANOVA (p<0.05). The value was significant (p=0.000) and was categorized as significant differences between groups. The fourth test was Duncan's post hoc test, shown in Figure 1.

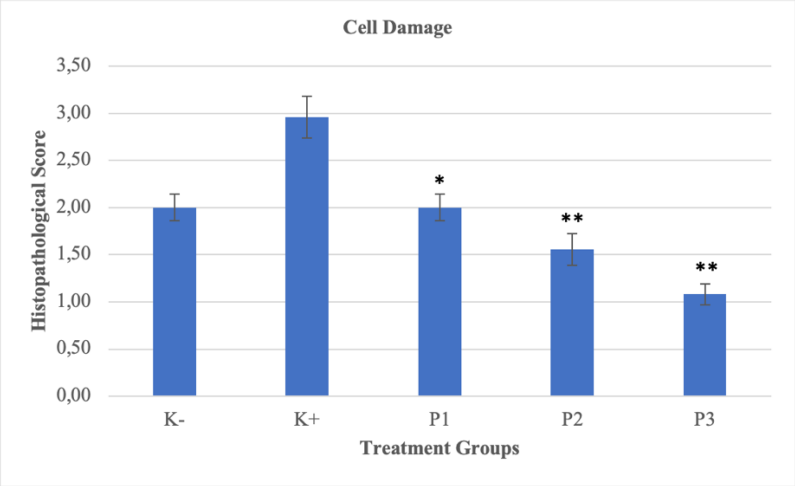
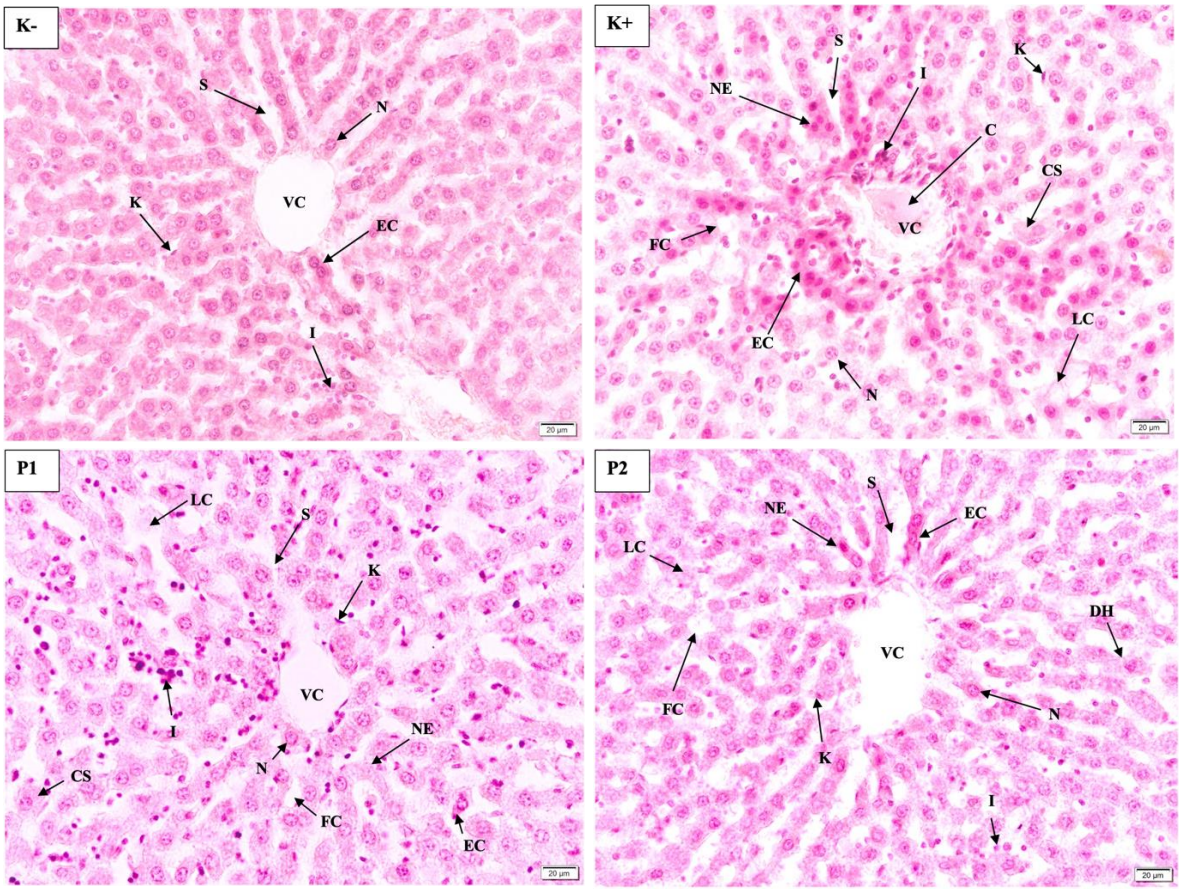


Figure 1. The graphic effect of the red okra pods' ethanol extract (ROE) to protect against DMBA-induced damage in rats' liver. K-: negative control (a single dose of 0.5 mL corn oil), K+: positive control (a single dose of 80 mg/kg BW dissolved in 0.5 mL corn oil), P1, P2, and P3: treatment groups 1, 2, 3 (a single dose of 80 mg/kg BW dissolved in 0.5 mL corn oil and ROE of 50, 100, and 200 mg/kg BW).
Legend: *Significantly different from K+ group.
**Significantly different from K+ and K- group (p>0.05).



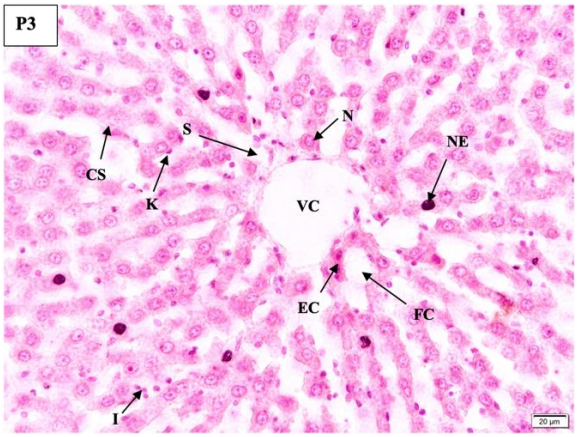


Figure 2. The microscopic structure of the liver stained by H&E (40x magnification) shows the effects of red okra pods' (ROE) ethanol extracts to protect against DMBA-induced damage in rats' liver histopathologically. K-: negative control (a single dose of 0.5 mL corn oil), suffering 35% from cellular damage; K+: positive control (a single dose induction of DMBA 80 mg/kg BW dissolved in 0.5 mL corn oil), suffering 51.8% from cellular damage; P1: treatment group 1 (a single dose of 80 mg/kg BW dissolved in 0.5 mL corn oil and ROE of 50 mg/kg BW), suffering 35% from cellular damage; P2: treatment group 2 (a single dose of 80 mg/kg BW dissolved in 0.5 mL corn oil and ROE of 100 mg/kg BW), suffering 27.3% from cellular damage; and P3: treatment group 3 (a single dose of 80 mg/kg BW dissolved in 0.5 mL corn oil and ROE of 200 mg/kg BW), suffering 18.9% from cellular damage. Legend: VC: vena centralis, N: normal, NE: necrosis, S: sinusoid, K: Kupffer cell, CS: cellular swelling, FC: fatty change, EC: eosinophilic cytoplasm, I: inflammation cell, LC: leakage of content, and C: congestion.

DISCUSSION

Effect of DMBA induction in the liver microscopic structure of rats

The cellular damage can be classified as reversible or irreversible. Reversible damage has the main morphologies of cellular swelling and fatty change. Meanwhile, irreversible damage has the main morphologies of necrosis, eosinophilic cytoplasm, inflammatory cells, and content leakage (Kumar, et al., 2017; Sijid, et al., 2020). Based on the results, K- showed 35% cellular damage in the liver. The inflammatory cells are visible, although not dominant, in the central vein. Some of the risk factors that promote a low-grade inflammatory response include age, obsession, dietary patterns, stress, and sleep disorders (Chen, et al., 2018). In addition, Sijid, et al., (2020) explained that the central vein is sensitized to toxic exposure because of endothelial cells. Besides, blood flows from the portal vein and hepatic artery are contained in the central vein, so if there is damage, the inflammatory cells of lymphocytes diffuse to the central area (Sijid, et al., 2020). Meanwhile, the induction of the DMBA group (K+) showed 51.8% of cellular damage in the liver. Plante (2021) explained that DMBA damages the organ by activating intracellular aryl hydrocarbon receptor (AhR) signaling to induce transcription of cytochrome P450 genes (CYPs) whose final metabolism is 7, 12-DMBA-3,4-diol-1,2-epoxide. The condition of the DMBA epoxide metabolite increases ROS, resulting in an imbalance in the body that will trigger oxidative stress (Plante, 2021). Swelling cells, fatty change, necrosis, eosinophilic cytoplasm, inflammatory cells, and content leakage are seen predominantly. Swelling cells, also known as hydropic degeneration, occur due to increased plasma membrane permeability that fails to maintain ionic and fluid homeostasis. Therefore, cells and intracellular organelles absorb water and then swell. Furthermore, fatty change occurs due to lipid metabolism, which causes the formation of lipid vacuoles as abnormal deposits of cytoplasm containing triglycerides. Besides, necrosis occurs due to severe damage to cellular components characterized by irregular liver cell shape, increased color in the eosinophilic cytoplasm, and cell nuclei damage. The necrosis cells have patterns of pyknosis, karyorrhexis, and karyolitic. The state of necrosis can cause dilatation of sinusoids. Sinusoids and liver cells are only limited by subendothelial-containing microvilli, so that contact exchange of compounds, including toxic compounds, becomes easy (Krishna, 2017; Kumar, et al., 2017; Sijid, et al., 2020). In addition, congestion conditions are predominantly seen in K+. Congestion can be characterized by

blood cells that fill the lumen in blood vessels (Sijid, et al., 2020). This condition is caused by oxidative stress that causes vascular damage, vasoconstriction of blood vessels, decreased blood flow, and increased blood dose and viscosity (Nurhidayat, et al., 2022). DMBA induction in the K+ group showed a significant increase in liver damage compared to K-. The results of the study were in line with research by Nurhidayat, et al., (2022) and Abdelmeguid, et al., (2021) which compared the control group and the DMBA induction group that experienced significant damage to the microscopic structure of the liver.

Effect of various doses of ethanol extract of ROE in the liver microscopic structure of rats

Plant antioxidants are important as natural reserves of bioactive compounds for plant adaptation, acclimation to environmental challenges, and being beneficial for human health (Maury, et al., 2020). Okra contains polyphenol components (29.5%), such as phenolic acids, flavonoids, and quercetin. Quercetin-3-O-gentiobioside is a major contributor to antioxidant capacity (Elkhalifa, et al., 2021). Based on the results, the administration of ROE in the P1, P2, and P3 showed 35%, 27.3%, and 18.9% cellular damage, respectively. Wahyuningsih, et al., (2020a) explained that the antioxidants in okra have several structures of hydroxyl groups that can donate H+ atoms to stabilize ROS by delaying or inhibiting damage. In addition, the study by Wahyuningsih, et al., (2020b) showed that okra pod extract has a value of 35.21 µg/mL, an inhibitory concentration (IC_{50}) in the 2, 2-diphenyl-picrylhydrazyl (DPPH) antioxidant activity test, so that it is categorized as a powerful antioxidant. These results indicate that the antioxidants in ROE doses of 50, 100, and 200 mg/kg BW could help the endogenous antioxidants balance with oxidants that trigger oxidative stress. The findings of this study were in line with a study by Wahyuningsih, et al., (2020a) who found that antioxidant okra pods methanol extract can repair damage to the microscopic structure of the liver in mice that hepatotoxicity induced by sodium nitrate. Specifically, it demonstrated a decreasing proportion of liver cells that experienced swelling, necrosis, and inflammatory cells. In addition, another study reported that seeds and peels of okra pods could increase levels of enzyme antioxidants endogenous superoxide dismutase (SOD) and glutathione peroxidase (GPx), and also have reduced levels of malondialdehyde (MDA) in rat models of diabetes caused by streptozotocin (Elkhalifa, et al., 2021).

Strength and limitations

This study has the potential to become a reference in red okra pods (*Abelmonchus esculentus* (L.) Moench) and 7, 12-dimethylbenz[a]anthracene (DMBA) for future studies. This research had limitations because the plant's high and low antioxidant activity can be influenced by several environmental factors, such as plant conditions (age, soil conditions, pathogen infection, and air) and exposure, such as oxygen, light, and high temperature. Therefore, the red okra fruit obtained should be screened for total phenolic and flavonoid calculations first. This aimed to determine the accurate amount of antioxidant content in the sample used.

CONCLUSION

The red okra pods' ethanol extract can protect against DMBA-induced damage in rats' liver. The red okra pod successfully stimulated a lowering histopathological score based on percentages of reversible and irreversible cellular damage in rat's liver histology, such as cellular swelling, fatty change, eosinophilic cytoplasm, inflammation cells, leakage of content, necrosis cells, and congestion. This suggests that red okra pod ethanol extract has antioxidant activity against exposure to oxidants by environmental pollutants in the form of DMBA.

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Conflict of Interest
All authors have no conflict of interest.

Ethic Consideration
The Health Research Ethical Clearance Commission of the Faculty of Dental Medicine, Universitas Airlangga, approved this study (Registration Number. 444/HRECC.FODM/VII/2022) on 20-07-2022.

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This study did not receive any funding.

Author Contribution
LDT contributed to the conception and design, analysis and interpretation of the data, and drafting of the article. KEP contributed to the conception and design, critical revision of the article, and final approval of the article. ISM contributed to the conception and design, critical revision of the article, and final approval of the article. SPA contributed to the provision of study materials, statistical expertise, and final approval of the article. NIL contributed to the final approval of the article.

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