Ethanolic root extract of *Bouea macrophylla* Griffith improves lipid homeostasis in palmitate-induced lipid accumulation in HepG2 cells

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ABSTRACT

The major characteristic of nonalcoholic fatty liver disease (NAFLD) is the excessive triglyceride accumulation in hepatocytes due to an imbalance between lipid intake and removal, which also disrupts other lipid metabolism pathways. Therefore, the present study explored the effect of *Bouea macrophylla* Griffith root ethanolic extract (BME) on lipid homeostasis in palmitate-induced steatosis in HepG2 cells as well as the phytochemical content of BME. In palmitic acid-induced lipogenesis in HepG2 cells, BME (5–10 µg/ml) could suppress the expression of lipogenic genes, including sterol regulatory element-binding protein 1c, acetyl-CoA carboxylase, fatty acid synthase, and reduced lipid storage. Interestingly, the expression of the fatty acid oxidation gene, peroxisome proliferator-activated receptor α, was upregulated, while that of cytochrome P450 2E1 was downregulated by BME. The screening of phytochemicals showed the presence of amines, flavonoids, and phenolics, and high-performance liquid chromatography analysis revealed gallic acid as the major bioactive component of BME. These findings indicate that BME may be useful for improving abnormal lipid homeostasis in metabolic disease-related NAFLD.

INTRODUCTION

Excessive storage of lipid in hepatocytes is the main characteristic of nonalcoholic fatty liver disease (NAFLD) [1] and is related to an increased risk of metabolic diseases such as obesity and dyslipidaemia [2]. The high level of free fatty acids (FFAs) in the blood is generally found in both obesity [3] and NAFLD [4]. Elevated FFA levels lead to increased uptake and storage of lipids in the liver and muscles [5]. This is accompanied by an increase in hepatic *de novo* lipogenesis and triglyceride synthesis mediated by sterol regulatory element-binding protein 1c (SREBP1c) [6]. Activation of transcriptional

factor SREBP1c stimulates main enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which contribute to excessive hepatic triglyceride accumulation in patients with NAFLD [7]. Peroxisome proliferator-activated receptor α (PPARα) is an important transcriptional regulator involved in hepatic lipid homeostasis, including fatty acid (FA) activation and transport to the mitochondria, β-oxidation, and lipogenesis [8]. Additionally, impaired β-oxidation may induce lipid accumulation. However, the activation of β-oxidation in the peroxisomes and microsomes is a possible pathway for regulating the overproduction of fatty acids (FAs) in hepatocytes [9]. Cytochrome P450 2E1 (CYP2E1) is highly expressed in response to the pathological processes of metabolic diseases [9]. CYP2E1 induction is an alternative response that prevents FA overload via β-oxidation [10], and elevated CYP2E1 activity has been reported in obesity and NAFLD [11]. Furthermore, the major FAs related to *de novo* lipogenesis are palmitic acid (PA) and stearic acid, which are linked with the risk of type 2

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diabetes and cardiovascular diseases [12]. It has been shown that PA can induce lipotoxicity in various cell lines [13–15], and HepG2 cells are also used to create the NAFLD model through PA induction [13,14,16,17].

Bouea macrophylla Griffith (BM), commonly known as plum mango, is a tropical Asian plant known as Maprang in Thailand [18]. This plant belongs to the mango family of Anacardiaceae. It has been reported that many parts of the BM, including the fruit, leaf, stem, and seed, have several pharmacological properties, such as antioxidant [19–21], anticancer [22–24], and antihyperglycaemic [25,26] activities. Moreover, the root extracts of some species in the Anacardiaceae family, including *Mangifera indica* (mango) and *Anacardium occidentale* (cashew), contain phenolic and flavonoid components with pharmacological effects such as antihyperglycaemia and antioxidation [27,28]. BM is also a member of the Anacardiaceae family; however, pharmacological information on its root part is still lacking, especially regarding its potential role in regulating obesity, which is one of the major public health threats [29]. Currently, medicinal plants are popular alternatives for disease treatment. Therefore, the present study was undertaken to examine the effect of BM root ethanolic extract (BME) on regulating lipid homeostasis in PA-induced lipid accumulation in HepG2 cells, which is related to various chronic diseases such as obesity and NAFLD.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased: standard bioactive compounds, including caffeic acid, chlorogenic acid, coumaric acid, ellagic acid, ferulic acid, gallic acid, hesperidin, quercetin, rosmarinic acid, rutin, and vanillin (Sigma-Aldrich, USA). Chemicals for cell culture and gene expression analysis were obtained as follows: sodium palmitate, bovine serum albumin (BSA) (FA-free), dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), and Oil Red O (ORO) dye (Sigma-Aldrich, USA), cDNA synthesis kit (Bio-Rad, USA), penicillin-streptomycin (Gibco, USA), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (Cytiva HyClone, USA).

Plant extraction

BM roots were collected from the Fruit Garden, Mueang District, Chanthaburi Province, Thailand. Voucher specimen, UBU-BM-1 (*B. macrophylla* Griffith, Thaweesak Juengwatanatrakul) was deposited in the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. The dried plant roots (30 g) were extracted with 300 ml of absolute ethanol for 3 days using the maceration technique, and the solvent was then evaporated to obtain the dry extract. The yield of dry powdered BME was 10%.

Phytochemical screening tests

Phytochemical screening of BME was performed to identify the main classes of compounds (alkaloids, amines, coumarins, flavonoids, and phenolics) present in the extracts, following standard protocols [30].

Total phenolic content (TPC) test

TPC was determined using the Folin-Ciocalteu assay, as described by Zahoor et al. [31] with slight modifications. BME (1 mg) was dissolved in methanol (1 ml). The prepared samples (1 ml) were then incubated with 10% Folin-Ciocalteu reagent (1 ml) and 7.5% sodium carbonate solution (2 ml) in the dark. Absorbance was measured after 30 minutes at 765 nm. TPC was calculated as mg gallic acid equivalents (mg GAE)/g of dry extract.

Total flavonoid content (TFC) test

BME (1 mg) was dissolved in methanol (1 ml) and diluted with distilled water (diluted 10-fold). The diluted samples were incubated with 5% sodium nitrite solution (2 ml) for 5 minutes and then 10% aluminium chloride solution (2 ml) was added. The mixture was vortexed and mixed with 1 M sodium hydroxide (2 ml) and after 10 minutes the absorbance was measured immediately at 415 nm. TFC was calculated as mg quercetin equivalents (mg QE)/g of dry extract.

High-performance liquid chromatography (HPLC) analysis

HPLC analysis was performed in triplicate by using a Dionex UltiMate™ 3000 HPLC system and C18 column (5 μm, 250 mm × 4.6 mm) (ACE, UK), following the protocol by Nanna et al. [32]. The mobile phase consisting of 0.1% acetic acid (solvent A) and acetonitrile (solvent B). The gradient set at 90:10 (A: B) for 5 minutes, shifted to 72:28 for 15 minutes, then to 50:50 for 10 minutes, followed by 35:65 for 10 minutes, 25:75 for 5 minutes, and finally to 0:100 for 5 minutes. The injection volume was 10 μl at a flow rate of 0.8 ml/minute and 25°C as the column temperature. The wavelength of detection was established at 254 nm. BME ingredients were identified by comparing their retention times and UV-VIS detector with those of the following standards (caffeic acid, chlorogenic acid, coumaric acid, ellagic acid, ferulic acid, gallic acid, hesperidin, quercetin, rosmarinic acid, rutin, and vanillin). Semi-quantitative data were analysed based on the area under the peak relative to the content of each component in the extract.

Cell culture and experimental design

All study protocol was reviewed and approved by the Thammasat University Institutional Biosafety Committee (TU-IBC 036/2566). HepG2 cells (Lot#70057473) were obtained from the American Type Culture Collection (Virginia, USA). An *in vitro* model of NAFLD was established by inducing lipid overload in hepatocytes through 24 hours incubation with a high concentration of PA [33]. The PA-BSA conjugate was prepared as previously described [34]. Briefly, a stock solution of PA (50 mM) was dissolved in 0.1 M NaOH, then diluted in DMEM containing 1% BSA (FA-free) and incubated for 1 hour to allow conjugation. The solution was filtered through a 0.22 μm filter and stored at −20°C until use. The PA stock was diluted with DMEM to a final concentration of 250 μM. HepG2 cells were divided into three groups: a control group (untreated), a PA-treated group, and a BME-treated group. Lipid overload was induced using 250 μM PA. After 24 hours of PA incubation, the various concentrations of BME (1, 5,

and 10 μg/ml) were added to the BME-treated groups for 48 hours. At the end of the experiment, cells were collected for lipid accumulation and lipogenic gene expression analyses. Six independent experiments were performed in duplicate.

Cell viability assay

HepG2 cells were seeded in a 96-well plate at a density of 1×10^4 cells/ml and cultured for 24 hours. Lipid overload was induced using PA, followed by treatment with various concentrations of BME (0, 1, 5, 10, 50, 100, and 200 μg/ml) for 48 hours. Then, 0.5 mg/ml MTT solution (100 μl) was added to each well and incubated at 37°C for 4 hours. The MTT solution was discarded, and 100 μl of DMSO was added to dissolve formazan crystals. Absorbance was measured at 545 nm to determine cell viability.

ORO staining

HepG2 cells were seeded at a density of 1×10^4 cells/ml in the culture chamber slides and 96-well plates. After 48 hours BME treatment, the medium was discarded and then rinsed with phosphate-buffered saline. Cells were fixed in 10% formalin and washed with isopropanol. Then, cells were stained with 0.6% ORO solution for 1 hour. A picture of stained cells was taken by a Primovert microscope (Carl Zeiss, USA) at ×40 magnification. Lipid accumulation in the culture plates was quantified by extracting the stain with isopropanol and measuring absorbance at 500 nm.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted according to the manufacturer's instructions (Vivantis, Kuala Lumpur, Malaysia), and RNA quantification was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was produced using the iScript cDNA synthesis kit. Reverse transcription polymerase chain reactionwas performed using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), with three technical replicates for each analysis. Primer sequences used in this study are listed in Table 1. The mRNA levels of all genes were normalised using β-actin as an internal control, and relative quantitation was performed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All experiments were performed in duplicate and repeated six times independently. Statistical analyses were performed using SPSS software (version 26.0). Results were expressed as mean ± SEM. Differences among groups were analysed using one-way ANOVA, followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

RESULTS

Phytochemical contents

The compound expression of alkaloids, amines, coumarins, flavonoids, and phenolic groups in BME was determined. The phytochemical content in the extracts was visually observed based on the color present. Results obtained are shown in Table 2, amines, flavonoids, and phenolics

Table 1. Primers and their sequences.

Primers	Primer sequences (5'- 3')
SREBP1c Forward	CCACTTCATCAAGGCAGACTCG
SREBP1c Reverse	CAAGATGGTTCCGCCACTCAC
ACC Forward	CTTGGCCTTGCACATAAGGTCC
ACC Reverse	CCACCTACGGATAGACCGCA
FAS Forward	ATAGTGTGGAAGACGCTGGC
FAS Reverse	CTGGTACACCTTCCCACTCAC
PPARα Forward	CAATGCACTGGAACTGGATGA
PPARα Reverse	GTTGCTCTGCAGGTGGAGTCT
CYP2E1 Forward	GCACAGGGACAGGGGAATC
CYP2E1 Reverse	GAGGAAGGTGGGGTCGAAAG
β-actin Forward	GATTCCTATGTGGGCGACGA
β-actin Reverse	AGGTCTCAAACATGATCTGGGT

Table 2. Phytochemical screening of BME.

Phytochemical	BME
1. Alkaloids	−
2. Amines	+ (purple)
3. Coumarins	−
4. Flavonoids	+ (orange)
5. Phenolics	+ (green)

Data are expressed as (+) for the presence and (−) for the absence of groups of compounds.

Table 3. Total phenolic content and total flavonoid content of BME.

Test	Content
1. Total phenolic content	616.18 ± 8.67 mg GAE/g
2. Total flavonoid content	142.65 ± 3.40 mg QE/g

Data are expressed as mean ± SEM ($n = 3$).
mg GAE = mg gallic acid equivalents; mg QE = mg quercetin equivalents.

were found in BME, while alkaloids and coumarins were not detected. The TPC and the TFC are shown in Table 3. The results indicated that phenolic compound (616.18 ± 8.67 mg GAE/g) was the most abundant in the extract, followed by TFC (142.65 ± 3.40 mg QE/g).

HPLC analysis

HPLC was employed for plant fingerprinting, and only gallic acid was quantified. The HPLC chromatograms of BME and gallic acid were shown in Figure 1A and B, respectively. BME showed that the gallic acid was predominantly present at 83.91 ± 0.01 mg/g of dry extract. No other standards were detected in the root extracts (Table 4).

Cell viability of BME

After 48 hours incubation with various BME concentrations, concentrations from 50 to 200 μg/ml of BME showed significant cytotoxicity (Fig. 2A). Thus, the present
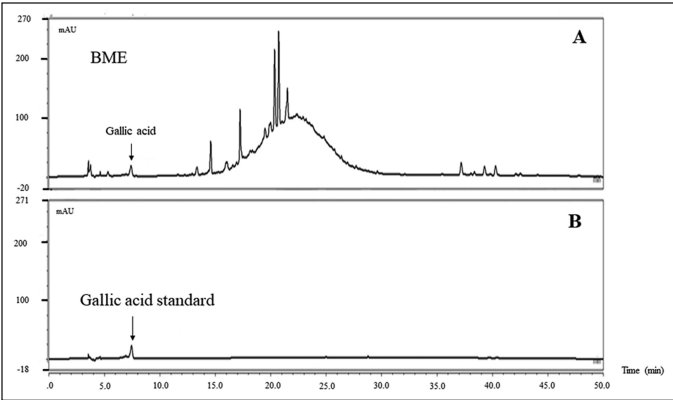
**Figure 1.** HPLC Chromatogram of BME (A) and gallic acid (B). Data are expressed as mean ± SEM ($n = 3$).

Table 4. Bioactive compound content of BME.

Bioactive compound	Content
1. Gallic acid	83.91 ± 0.01 mg/g dry extract
2. Caffeic acid	None
3. Coumaric acid	None
4. Ferulic acid	None
5. Rosmarinic acid	None
6. Chlorogenic acid	None
7. Ellagic acid	None
8. Vanillin	None
9. Rutin	None
10. Quercetin	None
11. Hesperidin	None

Data are expressed as mean ± SEM ($n = 3$).
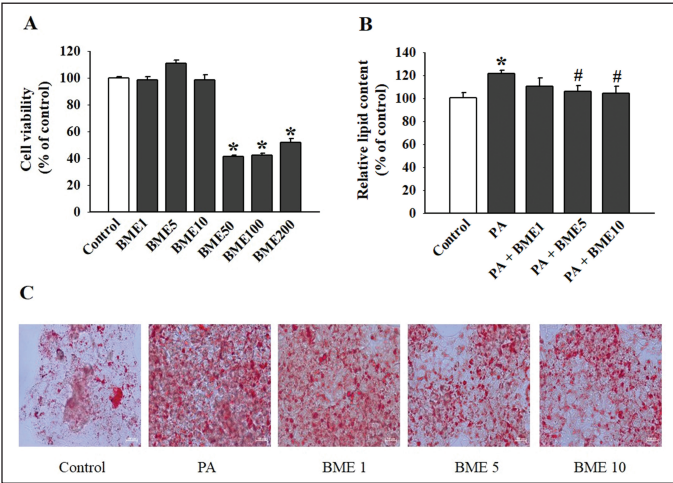
Figure 2. Effects of BME on lipid accumulation in PA-induced HepG2 cells. (A) Viability of HepG2 cells using MTT assay. (B) Lipid accumulation was extracted by isopropanol, and the quantitative content was measured at 500 nm. (C) Oil Red O-stained image of HepG2 cells observed under a microscope (400×). Data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$ versus the control group (untreated cells). #$p < 0.05$ versus the PA-treated group.
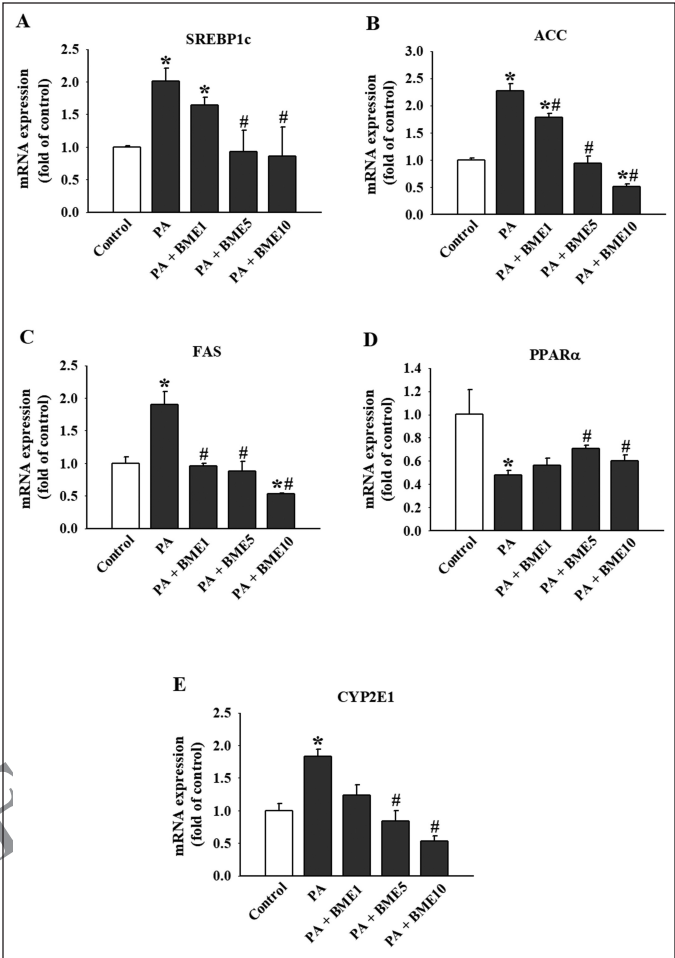
Figure 3. Effects of BME on gene expression of lipid homeostasis in PA-induced HepG2 cells. (A) SREBPIC, (B) ACC, (C) FAS, (D) PPARα, and (E) CYP2E1, β-actin was used as an internal control. Data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$ versus the control group (untreated cells). #$p < 0.05$ versus the PA-treated group.

study selected BME concentrations of 1–10 µg/ml for further examination of regulating impaired lipid homeostasis in HepG2 cells.

Hepatic lipid accumulation of BME

As shown in Figure 2B, the quantity of lipid droplets was significantly increased in the PA-treated group compared to that in the control group, whereas the BME (5–10 µg/ml) showed significantly decreased lipid accumulation. Moreover, the widespread lipid droplets were obviously revealed in the PA-treated group. Interestingly, the BME-treated groups could decrease lipid droplets in comparison to the PA-treated group (Fig. 2C).

Lipid homeostasis gene expression of BME

The PA-treated group showed significantly increased lipogenic gene expression of SREBP1c, ACC, and FAS compared to the control group (Fig. 3A–C). However, the BME-treated groups at 1–10 µg/ml significantly suppressed these genes in comparison to the PA-treated group. Furthermore,

compared to the PA-treated group, the concentrations of BME at 5 or 10 µg/ml significantly increased the expression of the FA oxidation gene, PPARα (Fig. 3D). Moreover, BME (5–10 µg/ml) significantly suppressed expression of the CYP2E1 gene (Fig. 3E).

DISCUSSION

An imbalance in lipid homeostasis is associated with obesity-related NAFLD [35,36]. Abnormal lipid accumulation associated with high circulating FFA levels results in an increased uptake of FFAs and lipid storage by hepatocytes, ultimately leading to hepatic steatosis [1]. Compared to the control group, the PA-treated group showed significantly increased lipid accumulation, upregulation of lipogenic genes (SREBP1c, ACC, and FAS), increased CYP2E1 expression, and suppressed expression of FA oxidation gene (PPARα). The transcription factor SREBP1c is required for the regulation of lipogenic genes involved in FA synthesis, such as ACC and FAS [37]. SREBP1c activation increases hepatic lipogenesis in NAFLD [38]. The results demonstrated that the induction of lipid accumulation by PA was a suitable model for investigating the regulation of lipogenesis. Therefore, we examined the effects of BME on PA-induced lipid accumulation in HepG2 cells. Our data revealed that BME treatment reduced lipid storage and suppressed the SREBP1c, ACC, and FAS expression. Therefore, decreased expression of lipogenic genes by BME could alleviate NAFLD pathogenesis. In addition, PPARα, a gene that is a main regulator of FA oxidation and can help protect against NAFLD progression [39,40], was upregulated by BME treatment. FA overload in hepatocytes can activate alternative pathways, such as CYP2E1-mediated ω-oxidation [10]. CYP2E1 activation has been reported in the PA-induced steatosis of HepG2 cells [16]. In addition, BME treatment decreased CYP2E1 expression. Our findings suggest that the administration of BME promotes hepatic FA oxidation via increasing the expression of PPARα. Therefore, the regulation of hepatic lipid accumulation by BME is essential for the treatment of fatty liver disease and related diseases, such as obesity, dyslipidaemia, and diabetes.

Several parts of BM contain high amounts of phenolic compounds and exhibit various pharmacological activities [19–24]. The phytochemical screening of BME showed the presence of amines, flavonoids, and phenols. TPC and TFC were also performed in BME. Similarly, root extracts from some species in the Anacardiaceae family, including *M. indica* and *A. occidentale*, contain phenolic and flavonoid components as well [27,28]. Furthermore, the HPLC chromatogram of BME quantified only gallic acid, at a concentration of 83.91 ± 0.01 mg/g dry extract. However, other standards (caffeic acid, chlorogenic acid, coumaric acid, ellagic acid, ferulic acid, hesperidin, quercetin, rosmarinic acid, rutin, and vanillin) were not detected in BME. From this evidence, gallic acid, which is found in BME, may be an active compound that improves impaired lipid homeostasis in HepG2 cells.

Gallic acid (3,4,5-trihydroxybenzoic acid; molecular weight 170.12 g/mol) is a natural phenolic compound found in several plants, including fruits and nuts [41]. Gallic acid has many pharmacological activities, including antioxidant [42,43] and anti-inflammatory effects [44–46]. Moreover, gallic acid has been reported to show anti-lipogenic activity in FA -induced HepG2 cells and in animal models of high-fat diet-induced obesity [41,47,48]. In agreement with our study, the anti-lipogenic action of gallic acid may be related to the ability of BME to inhibit lipid accumulation in PA-induced lipogenesis in HepG2 cells by suppressing lipogenic genes (SREBP1c, ACC, and FAS) as well as by activating FA oxidation via the PPARα gene. Moreover, BME suppressed CYP2E1 expression. Based on these results, we hypothesised that the high gallic acid content in BME may be responsible for its ability to control lipid homeostasis. Nevertheless, BME may contain additional bioactive compounds that could enhance or modulate its beneficial effects on lipid homeostasis. Therefore, further investigation is needed to identify and quantify these remaining bioactive constituents.

CONCLUSION

We investigated the effect of BME in an NAFLD model using PA-induced lipid accumulation in HepG2 cells. BME was found to inhibit lipid droplet formation and reduce the expression of lipogenic genes such as SREBP1c, ACC, and FAS, as well as suppress CYP2E1, while stimulating the expression of the PPARα gene in PA-induced lipid accumulation in HepG2 cells. These findings strongly support the beneficial effects of BME and its main bioactive compound, gallic acid, in regulating abnormal lipid homeostasis. Therefore, BME may serve as a useful natural alternative for treating metabolic disease-related NAFLD.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

FINANCIAL SUPPORT

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

Ethical approvals details are given in the 'Materials and Methods' section.

DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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