Phytochemical and Preliminary Pharmacological Evaluation of *Diospyros blancoi* Fruits

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ABSTRACT: *Diospyros blancoi*, a member of the Ebenaceae family known as 'Bilati gab' in Bangla, is traditionally used in treating a number of diseases. The study was aimed to isolate secondary metabolites from the fruits of *D. blancoi* and exploring the various pharmacological activities of the methanolic extract and its petroleum ether, chloroform and carbon tetrachloride fractions. Three compounds were obtained through phytochemical examination and identified as stigmasterol (**1**), lupeol (**2**) and betulinic acid (**3**) from the petroleum ether soluble fraction. In the castor oil-induced diarrhea model, methanolic extract and the petroleum ether fraction exhibited potent antidiarrheal activity with 42.04% ($p < 0.001$) and 36.94% ($p < 0.001$) defecation inhibition, respectively. The petroleum ether and carbon tetrachloride fraction showed mild to moderate efficacy in the hypoglycemic activity assay. In the tail flicking analgesic assay, methanol extracts and other fractions showed considerable central analgesic activity. In the acetic acid-induced writhing model, the petroleum ether fraction produced maximal ($p < 0.001$) peripheral analgesic properties. It is apparent from this study that *D. blancoi* fruits serve as a reservoir of potential bioactive molecules that might have novel analgesic or antidiarrheal activities, and further considerable investigations to find possible lead candidates are suggestive of this study.

Key words: *Diospyros blancoi*, stigmasterol, lupeol, betulinic acid, antidiarrheal, hypoglycemic, analgesic.

INTRODUCTION

Medicinal plants from nature meant to benefit mankind. People have been using plants for medical purposes throughout history.[1] The use of plants as therapeutic agents is a common phenomenon all over the world. Many countries have integrated traditional plant-based remedies into their national healthcare systems through regulatory measures. Many diseases ranging from mild to severe clinical conditions, such as fever, headaches, diarrhea, cough, constipation, skin problems, wounds, menstruation problems, rheumatism and cancer are treated with medicinal plants.[2-4] Medicinal plants are reservoirs of potent bioactive compounds, and the beginning of pure compound isolation from plants in the nineteenth century has advanced this discipline as the pure compounds can be used in a safer way with effective dosing.[4,5] A notable number of active constituents obtained from plant sources are already in clinical applications.[6]

Diospyros blancoi (*D. blancoi*), also known as *Diospyros discolor*, from the Ebenaceae family, is a widely distributed evergreen tree species, primarily found in monsoon-prone regions.[7] The *Diospyros* genus consists of more than 500 species.[8] Plants in the genus have been utilized in traditional medicine, and their health benefits are proven by extensive biological studies.[9,10] *D. blancoi* is a familiar plant in Bangladesh and the fruit of the plant is locally known as "Bilati gab".[11] Different parts of the plant are used in treating a number of disease conditions. Traditionally, the bark of *D. blancoi* has been used to

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cure fever, diarrhea, dysentery and cough. The fruits are also used as a gargle for aphthous stomatitis and to cure wounds.[12] Leaves and roots are used to manage skin problems like dermatitis and respiratory illnesses.[13] *D. blancoi* fruits are widely consumed, especially by pastoral people. The fruits are considered an important element ethnopharmacologically; they are given to patients in some regions for their wellbeing.[14] The large-scale usage of *D. blancoi* fruit in different disease conditions provoked its phytochemical screening.

The aim of this study is to investigate the phytochemicals and pharmacological properties of the fruits of *D. blancoi*. It was therefore, of interest to isolate and characterize the components from the *D. blancoi* fruits extract and investigate the antidiarrheal, hypoglycemic and analgesic properties of different extracts of the fruits. The findings would strengthen the knowledge about the medicinal properties of *D. blancoi* fruits and aid to develop strategies for the management of the diseases.

MATERIALS AND METHODS

Plant materials. *D. blancoi* fruits were collected from Manikganj district, Bangladesh. A taxonomist from the Department of Botany at Dhaka University substantiated the sample identification. The fruits (after being cut into small pieces) were sun-dried to make them ready for crushing. Coarse powder was retrieved from dried fruits using high-capacity milling machinery. The powder was stored in an appropriate condition for subsequent use.

Extraction and fractionation. The powder obtained from *D. blancoi* fruits (about 700 g) was taken in a round-bottomed flask, submerged with 2 liters of methanol. Periodic stirring was applied to the mixture for several days. It was subjected to filtration, and the desired crude extract was acquired by implementing the rotary evaporation technique on the filtrate at 40°C temperature. Solvent partitioning using the modified Kupchan approach was applied to the crude extract.[15] The methanolic crude extract (10 g) of *D. blancoi* fruits was agitated with 10% water in methanol to make a stock solution. The prepared solution was then fractionated using solvents of different polarities, namely petroleum ether, carbon tetrachloride (CCl_4) and chloroform ($CHCl_3$). All of the fractions went through the rotary evaporation procedure and the obtained materials were properly reserved.

Isolation and identification of compounds. After TLC screening, further phytochemical investigation of the petroleum ether portion was done. A gel permeation chromatography was conducted using Sephadex LH-20 as per table 1. Compounds **1** and **2** were obtained from petroleum ether fraction numbers 11 and 14, respectively, whereas compound **3** was isolated from fraction number 17. Three percent ethyl acetate in toluene was the mobile phase in case of compounds **1** and **2**. Compound **3** was obtained using the mobile phase of seven percent ethyl acetate in toluene.

Table 1. Gel permeation chromatographic solvent systems used for the analysis of the petroleum ether fraction of the methanolic extract of *D. blancoi* fruits.

Test tube no.	Solvent system in column chromatography	Proportion	Volume collected (ml)
1-20	n-Hexane: Dichloromethane: Methanol	2:5:1	50
21-30	Dichloromethane: Methanol	9:1	30
31-35	Dichloromethane: Methanol	1:1	20
36-40	Methanol	100%	20

Properties of the isolated compounds.

Stigmasterol (1). White Crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.35 (1H, m, H-6), 5.14 (1H, dd, *J* = 15.0, 6.5 Hz, H-22), 5.04 (1H, dd, *J* = 15.0, 9.0 Hz, H-23), 3.51 (1H, m, H-3), 1.00 (3H, s, Me-10), 0.92 (1H, s, H-20), 0.90 (3H, d, *J* = 6.5 Hz, Me-20), 0.84 (3H, d, *J* = 6.0 Hz, Me-25), 0.82 (3H, t, *J* = 6.5 Hz, Me-28), 0.80 (3H, d, *J* = 6.0 Hz, Me-25), 0.67 (3H, s, Me-13).

Lupeol (2). White gum; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.68 and 4.56 (each 1H, br. s, H_2-29), 3.20 (1H, dd, *J* = 5.03, 11.5 Hz, H-3), 2.28 (1H, m, H-19), 1.67 (3H, s, Me-20), 1.02 (3H, s, Me-8), 0.98 (3H, s,

Me-4), 0.95 (3H, s, Me-14), 0.82 (3H, s, Me-10), 0.79 (3H, s, Me-17), 0.76 (3H, s, Me-4).

Betulinic acid (3). Colorless amorphous power; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.67 (1H, br. s, H_a-29), 4.57 (1H, br. s, H_b-29), 3.13 (1H, dd, *J* = 11.2, 4.8 Hz, H-3), 3.02 (1H, m, H-19), 1. 69 (3H, s, Me-20), 1.00 (3H, s, Me-8), 0.97 (3H, s, Me-4), 0.95 (3H, s, Me-14), 0.86 (3H, s, Me-10), 0.75 (3H, s, Me-4).

Drugs and reagents. All the used experimental reagents and chemicals were of analytical grade. Normal saline solution (0.9% NaCl) was procured from Beximco Pharmaceuticals Limited, Dhaka; DMSO and Tween-80 were from Merck, Germany, and BDH Chemicals, Germany, respectively. The electronic balance machine was from Denver Instruments M-220, USA; the glucometer was from Braun, Germany; and the tubulin syringe with a ball-shaped end was from Merck, Germany. Castor oil and acetic acid were procured from local markets. Standard glibenclamide, loperamide and diclofenac sodium were obtained from Square Pharmaceuticals Limited, Bangladesh.

Experimental animals. To conduct the *in-vivo* investigations, Swiss-albino mice were used. The animals were of either sex (average weight of 30 g) and their age was nearly 4 to 5 weeks. Before the experiment, these mice were kept in polypropylene cages. Recommended conditions (temperature: 24±1°C; light and dark cycles: 12 h in succession) were ensured to give them a housing period. The experimental animals were fed with International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR'B)-formulated rodent food and water. Guidelines for the management of experimental animals were strictly followed throughout the investigations.[16]

Evaluation of antidiarrheal activity. The extracts of *D. blancoi* fruits were investigated to find their antidiarrheal properties using the castor oil-induced diarrhea model.[17] This assay was based on the diarrhea-inducing properties of ricinoleic acid, which is present in castor oil.[18] The animals were divided into six groups, with each group consisting of five mice. One group was fed with control (0.9% NaCl solution) and another group was fed with standard loperamide at a dose of 3 mg/kg. The methanolic extract and fractions (petroleum ether fraction, carbon tetrachloride fraction and chloroform fraction) of *D. blancoi* fruits were given to the remaining four groups at a dose of 400 mg/kg body weight. Thirty minutes after the administration, castor oil (1 ml) was administered orally to each animal. The fecal materials from each mouse were collected for 4 h. and the antidiarrheal activity of *D. blancoi* fruits extracts was interpreted with the help of the percentage defecation inhibition value. The percentage of defecation was enumerated by the following formula:

% Inhibition of defecation = (1-Fs/Fn) x 100

Where Fn = mean number of fecal pellets in the control group and Fs = mean number of fecal pellets in the sample-treated groups.

Evaluation of hypoglycemic activity. The plasma glucose-lowering ability of the methanolic extract and different fractions of *D. blancoi* fruits was estimated by a slightly changed version of the oral glucose tolerance test in mice.[19] Glibenclamide was used as the standard in the assay (at a dose of 10 mg/kg of body weight). At the beginning, the plasma glucose level of the experimental mice was measured. After 60 min, 120 min and 180 min of the administration of the control (0.9% NaCl solution), standard (glibenclamide) and different fractions of *D. blancoi* fruits, plasma glucose levels of the experimental animals were recorded to evaluate the hypoglycemic effect. A glucometer (Braun) along with glucose oxidase-peroxidase reactive strips was used for that purpose. The results obtained from the test sample groups were then compared with the results of the control group and the standard group to justify the hypoglycemic activity.

Evaluation of central analgesic activity. The central analgesic activity of *D. blancoi* fruits was studied using the previously reported tail-flicking method.[20] The activity was evaluated based on the time required by experimental mice to take back their tail from warm water because the time would be

longer in those animals treated with samples having analgesic properties. Six groups of experimental mice were made ready for this experiment, and four mice belonged to each group. NaCl solution (0.9%) was administered to a group as a control and diclofenac sodium at a 5 mg/kg dose as a standard was administered to another group. The test fractions of *D. blancoi* fruits were given to the remaining four groups at a dose of 400 mg/kg body weight. With the assistance of a mouse holder, every test animal had its tail submerged in hot water (55±2°C), and the amount of time it took for the tail to withdraw from the water was timed at 30, 60 and 90 min. To interpret the central analgesic activity, the percent time elongation of tail flicking was calculated by comparing the tail flicking times observed in the test sample groups to those of the control group.

$$\% \text{ Time elongation} = (1 - Ts/Tc) \times 100$$

Where Ts = mean tail flicking time of the mice in the sample group and Tc = mean tail flicking time of the mice in the control group.

Evaluation of peripheral analgesic activity. The peripheral analgesic efficacy of different fractions of *D. blancoi* fruits was determined using the widely used method in which the numbers of writhing in experimental animals are counted after acetic acid administration.[21] To make the experimental animals feel pain, acetic acid is given intraperitoneally. Consequently, the animals begin to writhe or squirm their bodies at regular intervals in an attempt to release their pain. Analgesic substances are thought to reduce the amount of writhing in animals. Thus, the writhing inhibition percentage value with respect to the control group was used to interpret the analgesic activity. At the beginning, the test samples (*D. blancoi* fruits' methanolic extract and other fractions), the control (0.9% NaCl solution) and the standard (diclofenac sodium) were administered orally to the experimental mice. Acetic acid (0.7%) was administered intra-peritoneally to each of the animals after 30 min (the time period for proper absorption) of previously administered samples. The number of squirms or writhing was counted for each mouse for a 10-min time period, starting after 5 min of the administration of acetic acid. The percentage inhibition of writhing was determined utilizing the following equation:

$$\% \text{ Inhibition of writhing} = (1 - Ws/Wc) \times 100$$

Where Wc = mean writhing number (control) and Ws = mean writhing number (sample)

Statistical analysis. The results obtained from all biological investigations were included for statistical analysis. The data was organized as mean ± SEM values. In the study, one-way analysis of variance (ANOVA), followed by Dunnett's test, was used to conclude the statistical significance of the collected data. The values of $p < 0.001$, $p < 0.01$ and $p < 0.05$ were considered statistically significant. Microsoft Excel (version 2010) and the Statistical Package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY) software were used to conduct the statistical analyses.

RESULTS AND DISCUSSION

Characterization of isolated compounds. A total of three compounds (**1**-**3**) were isolated from the petroleum ether-soluble portion of methanolic crude extracts of *D. blancoi* fruits by chromatographic separation and preparative TLC techniques (Figure 1). Comprehensive ^{1}H NMR spectral analysis was carried out to validate their identification. In addition, comparison with previously reported spectral data indicated that the isolated compounds were stigmasterol [22,23], lupeol[24] and betulinic acid.[25]

Antidiarrheal activity. The methanolic extract of *D. blancoi* fruits and the petroleum ether fraction exhibited significant antidiarrheal activity in the castor oil-induced diarrhea model with 42.04% ($p < 0.001$) and 36.94% ($p < 0.001$) defecation inhibition, respectively, at 400 mg/kg body weight dose. The standard loperamide showed an inhibition value of 47.77% ($p < 0.001$). The chloroform fraction and the carbon tetrachloride fraction of fruit produced 16.56% ($p < 0.05$) and 23.57% ($p < 0.01$) defecation inhibition, respectively (Figure 2). It has been reported that the presence of phytochemicals like tannins, alkaloids, saponins, flavonoids and sterols in plant materials is responsible for decreasing intestinal

motility and thus producing antidiarrheal effects.[26] The antidiarrheal activity shown by the different extracts might be due to the existence of these types of compounds in *D. blancoi* fruits.

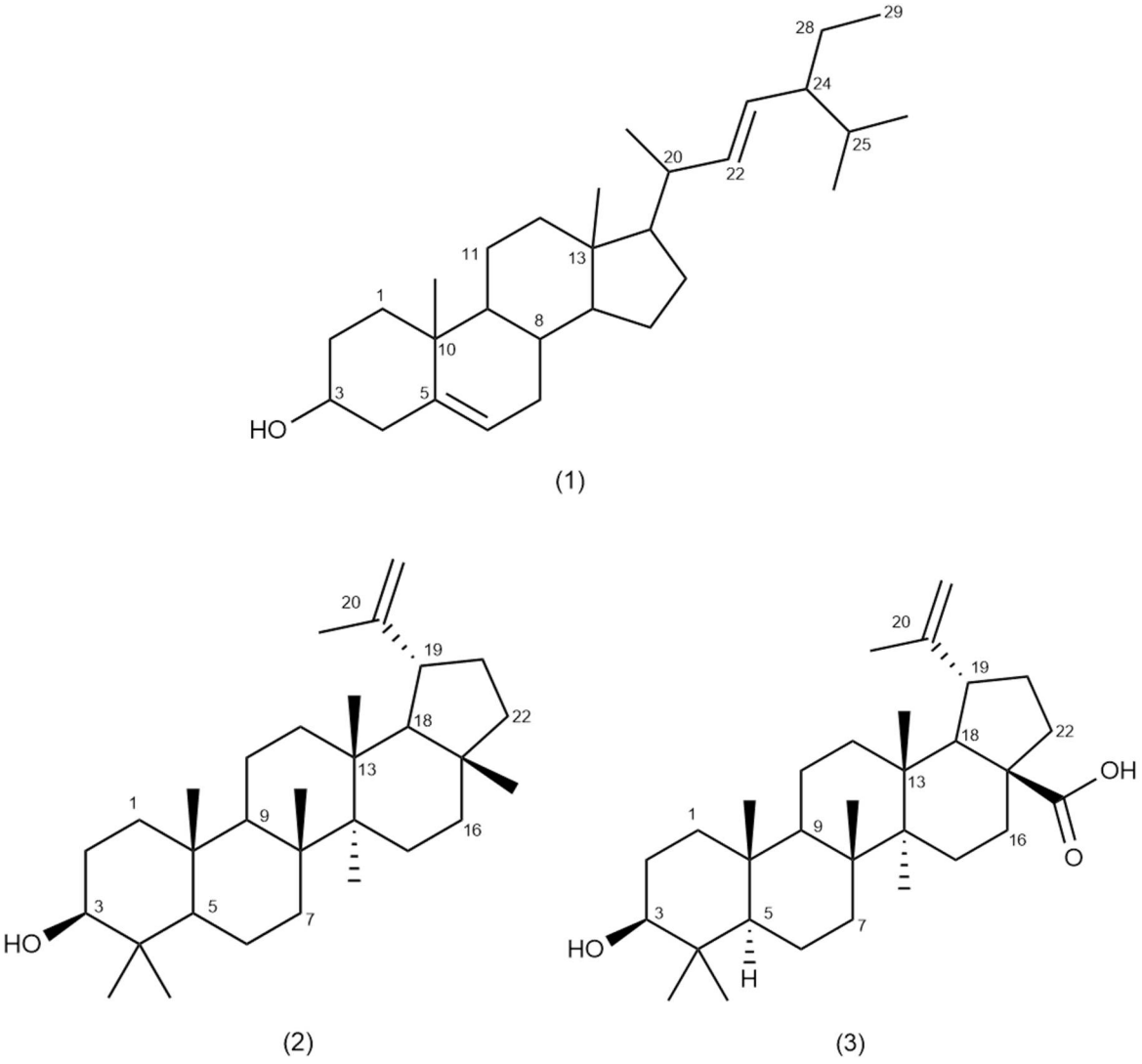

Figure 1. Structures of isolated compounds stigmasterol (**1**), lupeol (**2**) and betulinic acid (**3**).

Many of the currently used antidiarrheal medications are becoming resistant.[27] It has revitalized the interest of scientists in natural product-based drug discovery. The findings of the antidiarrheal activity assay revealed *D. blancoi* fruits as a potential base for research on antidiarrheal agents.

Hypoglycemic activity. The hypoglycemic activity of the test samples was monitored by their blood glucose-lowering ability in the experimental animals at different time intervals. The drug glibenclamide at a 5 mg/kg dose was the standard in this assay. Table 2 represented the recorded mean blood glucose levels obtained from the test sample-treated mice at 60, 120 and 180 min following administration. The standard sample reduced the blood glucose level from 6.08 mmol/L to 2.08 mmol/L after 60 min. The reduction percentage of

blood glucose level over time is presented in figure 3. Among the tested samples, the petroleum ether and carbon tetrachloride fractions of *D. blancoi* fruits exhibited statistically significant glucose-lowering effects after 60, 120 and 180 min. Overall, the findings of the oral glucose tolerance test claim that a mild to moderate hypoglycemic effect can be attained from *D. blancoi* fruits.

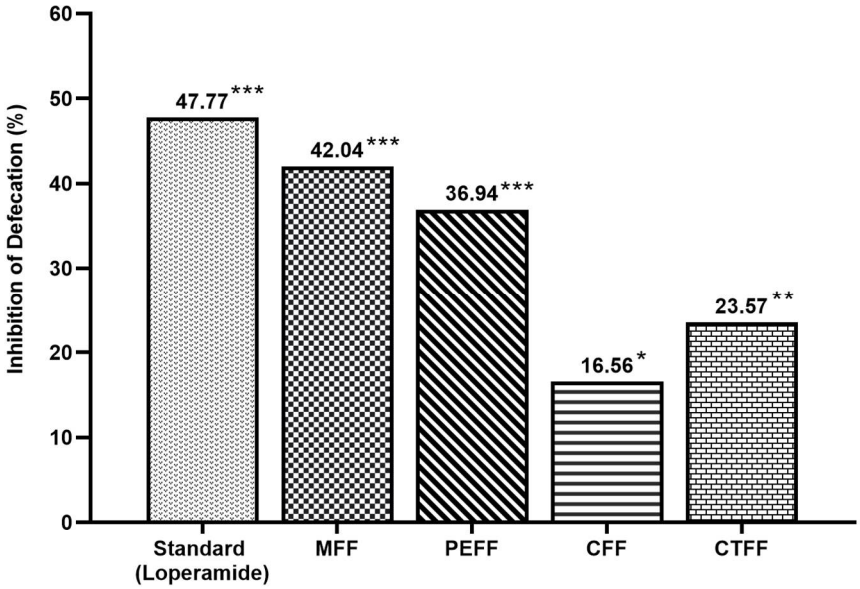

Figure 2. Antidiarrheal effect of methanolic extract and different fractions of *D. blancoi* fruits. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. [MFF = Methanol fraction of fruits, PEFF = Petroleum ether fraction of fruits, CFF= Chloroform fraction of fruits, CTFF = Carbon tetrachloride fraction of fruits]

Table 2. Blood glucose levels in mice treated with methanolic extract and different fractions of *D. blancoi* fruits.

Groups	Mean plasma level of glucose (mmol/L)			
	0 minutes	60 minutes	120 minutes	180 minutes
Control	6.40 ± 0.23	6.23 ± 0.11	5.63 ± 0.26	5.53 ± 0.15
Standard (5 mg/kg)	6.08 ± 0.17	2.08 ± 0.14	2.63 ± 0.17	2.73 ± 0.18
MFF (400 mg/kg)	7.98 ± 0.90	7.38 ± 1.55	6.15 ± 0.18	5.18 ± 0.20
PEFF (400 mg/kg)	7.40 ± 0.11	5.78 ± 0.13	5.65 ± 0.17	5.28 ± 0.18
CFF (400 mg/kg)	6.80 ± 0.45	6.30 ± 0.19	6.18 ± 0.23	5.93 ± 0.11
CTFF (400 mg/kg)	7.43 ± 0.17	6.20 ± 0.23	6.63 ± 0.19	5.78 ± 0.17

Values are expressed as Mean ± SEM (n = 4)

Central analgesic activity. The percentage of elongation time of the test sample groups and standard group after 30, 60 and 90 min has been presented in figure 4. Among the test sample groups, the methanolic extract of *D. blancoi* fruits at a dose of 400 mg/kg of body weight exhibited the highest efficacy, with 178.10%, 282.55%, and 331.89% time elongation values after 30, 60 and 90 min, respectively and the values were statistically significant ($p < 0.001$). The petroleum ether fraction also exhibited potent analgesic activity at 400 mg/kg of body weight dose with 160.86% ($p < 0.001$), 248.99% ($p < 0.001$) and 285.45% ($p < 0.001$) time elongation after 30, 60 and 90 min. The findings of this assay demonstrated the potential central analgesic activity of *D. blancoi* fruits.

Peripheral analgesic activity. In screening the peripheral analgesic properties of the methanolic extract of *D. blancoi* fruits and other fractions, diclofenac sodium (5 mg/kg) was given to the

experimental mice as the standard. The standard group showed 59.12% ($p < 0.001$) writhing inhibition (Figure 5). At a dose of 400 mg/kg of body weight, the petroleum ether fraction had the most prominent analgesic effect (55.97% writhing inhibition) among the test samples, which was statistically significant ($p < 0.001$). The methanolic extract showed 44.65% ($p < 0.001$) writhing inhibition, which can be claimed to possess strong analgesic activity. The chloroform fraction of the *D. blancoi* fruits also exhibited good peripheral analgesic properties, with 36.48% ($p < 0.001$) writhing inhibition at 400 mg/kg of body weight dose.

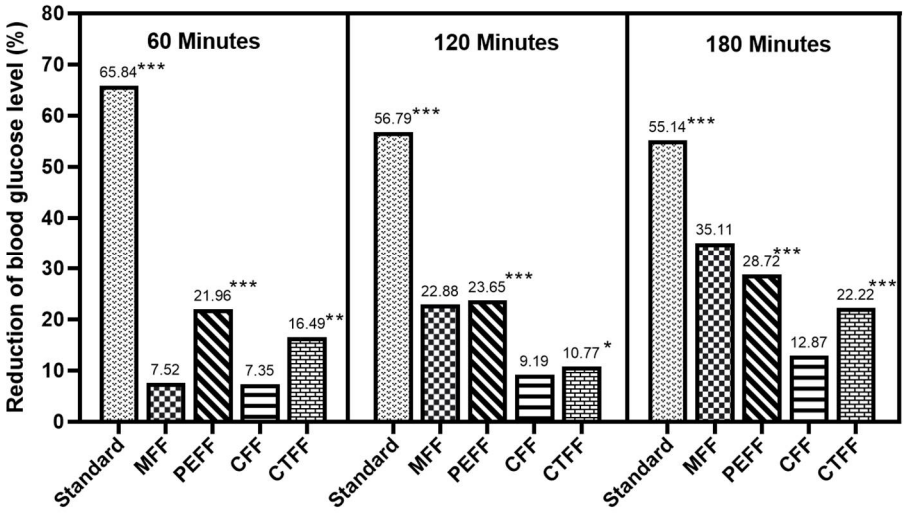

Figure 3. Hypoglycemic activity of the methanolic extract and different fractions of *D. blancoi* fruits. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. [Glibenclamide was used as standard, MFF = Methanol fraction of fruits, PEFF = Petroleum ether fraction of fruits, CFF= Chloroform fraction of fruits, CTFF = Carbon tetrachloride fraction of fruits]

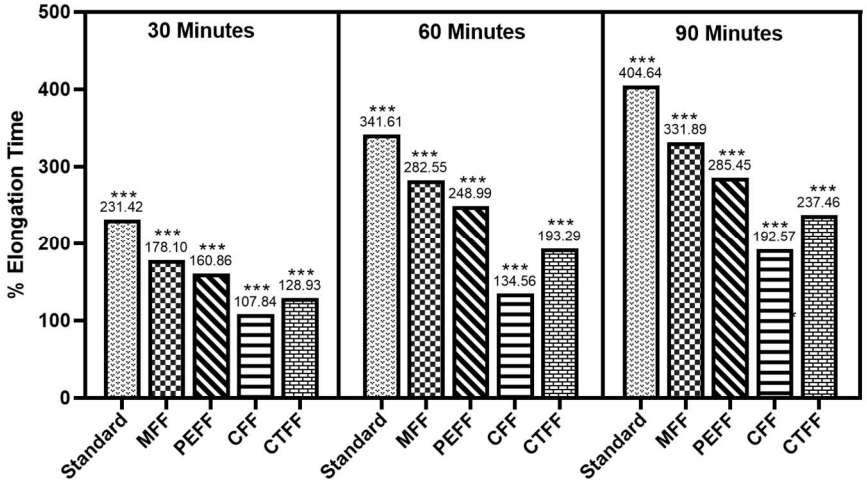

Figure 4. Central analgesic activity of different extracts of *D. blancoi* fruits. ***, $p < 0.001$. [Diclofenac sodium was used as standard, MFF = Methanol fraction of fruits, PEFF = Petroleum ether fraction of fruits, CFF= Chloroform fraction of fruits, CTFF = Carbon tetrachloride fraction of fruits].

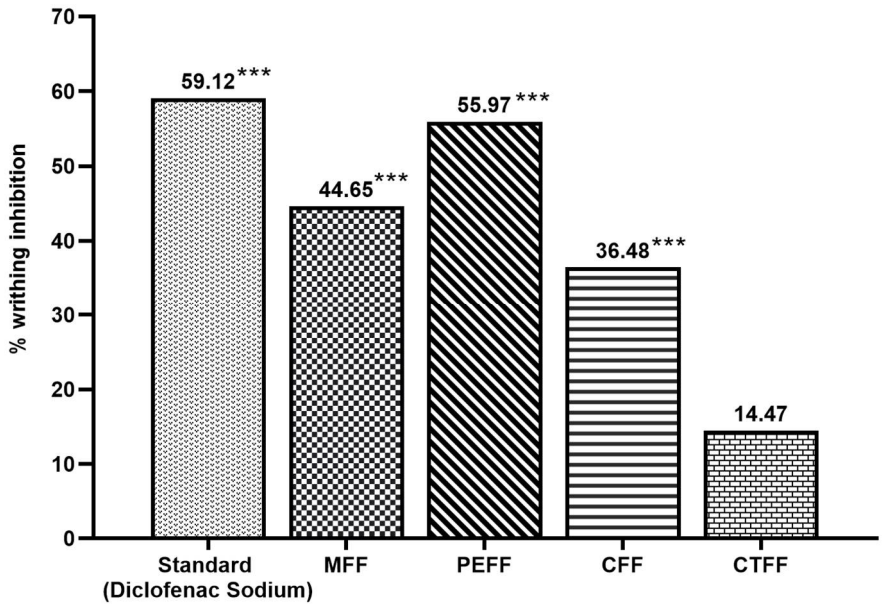

Figure 5. Peripheral analgesic effect of methanolic extract and different fractions of *D. blancoi* fruits; ***p < 0.001. [MFF = Methanol fraction of fruits, PEFF = Petroleum ether fraction of fruits, CFF= Chloroform fraction of fruits, CTFF = Carbon tetrachloride fraction of fruits].

The analgesic effects of *D. blancoi* fruits were evaluated by the tail flicking method and the acetic acid-induced writhing method, two well-established *in vivo* assays to justify the pharmacological activity. The centrally acting analgesics work by either resetting the physiological response to pain sensation or increasing the tolerance level.[28] The findings from two assays showed potent analgesic properties, indicating the possibility of utilizing both peripheral and central mechanisms of operation by the phytochemicals present in *D. blancoi* fruits, primarily in methanol extracts and pet ether fractions.

A number of compounds with reported analgesic activity were found in other parts of *D. blancoi*, including squalene and amyrin derivatives.[29] Besides, the presence of steroids, alkaloids and flavonoids can be considered to be a possible reason for the analgesic activity, as these groups of compounds are capable of producing analgesic actions and the presence of such compounds has been reported in other plants from the *Diospyros* genus.[30,31] *D. blancoi* fruits may contain these types of bioactive compounds, thus aggravating further phytochemical research.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are broadly prescribed medications in pain management, but these medicines are not safe and have a lot of side effects.[32] It has led to an increase in demand for the development of safe and more effective analgesics. The fruits of *D. blancoi* appear to be of potential interest, considering their analgesic properties.

CONCLUSION

Phytochemical investigation of *D. blancoi* friuts led to the isolation of three compounds identified as stigmasterol (**1**), lupeol (**2**) and betulinic acid (**3**). The findings of the preliminary pharmacological assays suggest that *D. blancoi* fruit exhibited a wide variety of biological activities, such as antidiarrheal, analgesic and hypoglycemic effects. The findings of the preliminary pharmacological assays clearly suggest that *D. blancoi* fruits serve as a reservoir of potential bioactive molecules that might have novel analgesic or antidiarrheal activities. So further considerable investigations are necessary for the development of safer drugs.

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