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Acute Oral Toxicity and Antispasmodic Effects of Two Extracts of *Diospyros mespiliformis* Hochst. Ex A. DC. (Ebenaceae) Immature Fruits on the Isolated Rat Duodenum

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Our study aimed to evaluate the chemical profile, the antioxidant and anti-inflammatory activities, the spasmolytic effects, and the safety of use of hydroethanolic extract (HE_FDM) and aqueous macerate extract (AM_FDM) of *Diospyros mespiliformis* immature fruits.
Place and Duration of Study: Immature fruits of *Diospyros mespiliformis* were collected in August 2022 in Goundi, in the Sanguié Province of Burkina Faso. The experiments were conducted in September - December 2022 at Laboratoire de Recherche-Développement de Phytomédicaments et Médicaments/IRSS/CNRST, Ouagadougou.
Methodology: Phytochemical screening by HPTLC and determining total polyphenols and flavonoids were carried out. The antioxidant activity was evaluated using DPPH, ABTS, FRAP, and LPO methods. The inhibitory activity of 15-lipoxygenase and Phospholipase A2 was evaluated. The *ex vivo* antispasmodic effect of extracts was tested on isolated rat duodenum using ACh and $BaCl_2$ as contracting agents. Finally, acute oral toxicity was carried out on female mice (NMRI).
Results: At the end of these tests, the extracts contain tannins, flavonoids, sterols, triterpenes, and saponosides. HE_FDM gave the highest levels of total phenolics (71.32±2.26 mg TAE/g and flavonoids (16.37±0.24 mg QE/g). Of the two extracts, HE_FDM gave the best antioxidant activity. However, these activities were lower than the reference substances ($p<0.05$). The extracts have a moderate inhibitory effect on phospholipase A2 and 15-Lipoxygenase. In addition, the most active HE_FDM caused a spasmolytic effect with Emax of 98.87±5.14% and 81.61±7.56%, respectively, during contractions induced by $BaCl_2$ and ACh. Finally, the extracts are practically non-toxic.
Conclusion: This work provided scientific data and could justify the use of *D. mespiliformis* immature fruits in the treatment of gastrointestinal disorders.

Keywords: Diospyros mespiliformis; immature fruits; antioxidant; antispasmodic; safety of use.

1. INTRODUCTION

"Gastrointestinal disorders are one of the most common illnesses in humans. These most common conditions affect the greatest number of children and adolescents" [1]. "To deal with these gastrointestinal disorders, people regularly turn to alternative medicine. Traditional medicine remains the first line of health care for 80% of the population in developing countries" [2]. "Plants are a widely exploited source of bioactive compounds in alternative and modern medicine. They constitute a useful matrix for identifying phytochemical compounds, which are then optimized by the pharmaceutical industry" [3]. "Burkina Faso offers a great botanical treasure trove, given its geographical and climatic diversity, and is a major source of potentially therapeutic plants for the treatment of diseases with gastrointestinal spasmodic components" [4]. "Antispasmodics or spasmolytics are drugs used to treat muscle spasms, which are intense and sudden pathological contractions of involuntary or smooth muscles" [5]. "On a pre-clinical level, ethnobotanical, biochemical, pharmacological, and toxicological studies and clinical trials have been carried out to reinforce this cultural heritage and provide scientific evidence" [6, 7]. "Given this cultural dimension, it is vital to enhance the value of medicinal plants by stepping up research into plant species widely used for their medicinal and especially spasmolytic properties. Among these potentially antispasmodic plants is *Diospyros mespiliformis* Hochst ex A. DC (Ebenaceae)" [8]. The literature tells us that extracts of *Diospyros mespiliformis* trunk bark have antiproliferative properties [9]. Leaves extracts have antioxidant and antimicrobial properties [8, 10]. The anti-plasmodial activity of methanolic fractions and extracts of trunk bark and leaves has also been demonstrated [8, 11]. Methanol extract from the trunk bark of *Diospyros mespiliformis* has been evaluated for its claimed popular use in the relief of fever, pain, and inflammation using Wistar rats and Swiss albino mice [12]. The fruits of *Diospyros mespiliformis* used in the treatment of dysentery [13] contain tannins and saponosides which may be responsible for the effect. The analgesic, antipyretic, and anti-inflammatory effects of the extract were evaluated in rats and mice. In

addition, the anti-inflammatory and spasmolytic properties of leaves have recently been demonstrated [4]. However, given the traditional use of the fruits of this plant, the present study was carried out to examine the safety and possible antispasmodic activity of the aqueous and hydroethanolic extracts of the immature fruits on acetylcholine (ACh)- and $BaCl_2$-induced contractions of the isolated rat duodenum.

2. MATERIALS AND METHODS

2.1 Plant Material

Immature fruits of *Diospyros mespiliformis* Hochst. ex A. DC (Ebenaceae) were collected in May 2022 in Goundi, in the Sanguié Province of Burkina Faso. One sample (4267 OUA) was identified and authenticated by a botanist from the Plant Biology and Ecology Laboratory at Joseph KI-ZERBO University, Burkina Faso. Fruits cut into small pieces were dried in the shade, away from direct sunlight and with ventilation. After drying, the fruits were pulverized using a mechanical grinder (Gladiator Est. 1931 Type BN 1 Mach. 404611083) to obtain a dry extractive powder.

2.2 Preparation of Immature Fruits Extracts

Extraction was carried out using the maceration exhaustion technique [14]. One hundred (100) g of immature fruit powder was macerated in 500 mL of distilled water or 80% ethanol (absolute ethanol/water; 80/20; v/v) for 24 h at room temperature. After 24 h, each residual pomace was repeated twice under the same conditions with distilled water or 80% ethanol. The two supernatants were filtered through fine mesh nylon cloth and centrifuged at 2000 rpm for 5 min. The hydroethanolic filtrate was concentrated in a rotavapor (ventilated Electronic Microprocessor Controller CPS) under reduced pressure at a constant temperature of 60°C, then frozen and freeze-dried. The filtrate of the aqueous macerate was frozen and freeze-dried. The hydroethanolic extract (HE_FDM) and the aqueous extract (AM_FDM) were then recovered and stored in two hermetically sealed bottles for the various tests.

2.3 Phytochemical Investigation: High-Performance Thin-Layer Chromatography

High-performance thin-layer chromatography (HPTLC) was used to detect flavonoids, tannins, sterol/triterpenes, and saponosides in the two extracts (HE_FDM and AM_FDM). “It was carried out on chromatoplates (60 F_{254}, 10 x 5 cm, glass support 10 x 20 cm, Macherey-Nagel, Germany Merck) following the literature [3, 4]. Approximately 20 µL of each extract was streaked with a semi-automatic sample dispenser (CAMAG, Linomat 5, Switzerland) along the baseline 0.8 cm from the bottom edge of the plate. After deposition and drying, the plates were placed in a tank containing eluent previously saturated (20×10 cm, saturation time: 30 min). The solvent system used depended on the metabolite to be identified: ethyl acetate/formic acid/H_2O (18/2/4/2/1 v/v/v/v) for tannins, ethyl acetate/formic acid/H_2O, (8/2/1 v/v/v/) for flavonoids, ethyl acetate/hexane (8/2 v/v) for sterol-triterpenes and hexane/ethyl acetate/methanol (10/5/5 v/v/v) for saponosides. After migration over 0.8 dm in length, the plates were dried, and sulphuric anisaldehyde reagent for saponosides, Neu reagent for flavonoids, Liebermann and Burchard reagent for Sterol-triterpenes and 5% $FeCl_3$ for tannins revealed the chromatographic profiles. The profiles were then observed under visible light (tannins)”.

2.4 Determination of Total Phenolic Compounds

The total phenolics content of HE_FDM and AM_FDM were determined using the Folin-Ciocalteu Reagent (FCR) [14]. The reference compound (Gallic acid) was used as to produce the standard curve. Twenty-five (25) µL of sample at a concentration of 1 mg/mL was mixed with 125 µL of FCR. A volume of 100 µL of 7.5% w/v sodium carbonate solution was added to the mixture. After one hour, absorbance at 760 nm was measured using a microplate reader (Spectro UV, Epoch Biotek, USA). Results were expressed as mg Gallic acid equivalent (GAE)/g dry extract.

2.5 Determination of Flavonoid Compound

“Flavonoid content was assessed using an aluminum chloride reagent” [14]. “A standard calibration curve was plotted with Quercetin. One hundred (100) µL of HE_FDM or AM_FDM (1 mg/mL) were mixed with 100 µL of a 2% w/v Aluminium trichloride solution. After 10 min, absorbance was measured using a mass spectrophotometer. Absorbance at 415 nm was measured using a spectrophotometer (Epoch Biotek, USA) after 10 min. Results were

expressed as mg Quercetin equivalent (QE)/g dry extract". [14]

2.6 Assessment of Antioxidant Properties

2.6.1 DPPH• Essay

"Free radical scavenging activity of HE_FDM, AM_FDM, and Trolox was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as previously described" [15]. "The absorbance of 10 µL of samples and Trolox added to 200 µL of DPPH (0.04 mg/mL) was measured at 490 nm after 30 min of incubation in the dark at room temperature using a Bio-Rad spectrophotometer (model 680, Japan). The result was expressed as antioxidant capacity equivalent to Trolox. As a function of sample concentration, a DPPH•-inhibition percentage curve was plotted" [15]. The percentage inhibition of the DPPH radical was calculated using the following formula:

Inhibition (%) = [(Ac - Ae)/Ac]x100

Ae and Ac represent the absorbances of the extract/ascorbic acid and the control (DPPH solution without sample). The concentration required to inhibit 50% of DPPH (IC_{50}) was determined on the curve.

Anti-radical power (ARP) was determined by the formula: ARP = $1/IC_{50}$; ARP: Anti Radical Power; IC_{50}: 50% inhibitory concentration expressed in µg/mL

2.6.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed on HE_FDM, AM_FDM, and Trolox as previously described [4]. The mixture of 0.5 mL samples with 1.25 mL phosphate buffer and 1.25 mL potassium hexacyanoferrate aqueous solution (1%) was incubated for 30 min at 50 °C. Next, 1.25 mL of trichloroacetic acid (10%) was added and centrifuged at 3000×g for 10 min. $FeCl_3$ solution (0.125 mL, 0.1%), and distilled water (0.625 mL) were added to the supernatant (0.625 mL). The absorbance of the reaction medium was measured at 700 nm using a spectrophotometer (Agilent, Santa Clara, CA) equipped with ChemStation UV-visible software. Trolox was used to plot the calibration curve. The FRAP activity of HE_FDM and AM_FDM was expressed as mol Trolox equivalent/gram dry extract.

2.6.3 ABTS•$^+$ Assay

"The ABTS free radical scavenging activity of HE_FDM, AM_FDM, and Trolox was assessed using the procedure described previously" [14]. "In a volume of 5 mL of distilled water, 19.2 mg of ABTS were dissolved. Potassium persulphate (3.312 mg) was added to the ABTS solution (3.84 mg/mL). After adding the potassium persulphate, the solution was left for 16 h in the dark at room temperature before use. On the day of the experiment, 4.5 mL of the mixture was diluted in 220 mL of absolute ethanol. The range of 8 dilutions to be tested was prepared from the parent concentration of the samples (1 mg/mL). On a 96-well microplate, 200 µL of ABTS solution mixed with 20 µL of HE_FDM or AM_FDM or Trolox were added to each well. After incubation for 30 min at 25 ºC, absorbances were read against a blank at 415 nm using an Agilent 8453 spectrophotometer with ChemStation UV-visible software. Measurements were performed in triplicate". [14] The percentage inhibition of absorbance at 415 nm was calculated according to the formula:

% Inhibition = $[(A_0\text{-}A)/ A_0] \times 100$

A_0 is the absorbance of the control; A is the absorbance of the sample.
The absorbance inhibition curve as a function of the concentration of the extract or reference substance (Trolox) was constructed to determine the 50% inhibitory concentration (IC_{50}). Anti-radical power (ARP) was determined using the formula: ARP = ($1/CI_{50}$); ARP: Anti-radical power; IC_{50}: 50% inhibitory concentration expressed in µg/mL

2.6.4 Lipid Peroxidation Inhibition (LPO) assay

"The lipid peroxidation activity of rat liver was determined using 2-thiobarbituric acid (Sigma Aldrich)" [14]. $FeCl_2$-H_2O_2 was used to induce peroxidation of liver homogenate. A 0.2 mL volume of HE_FDM or AM_FDM (1.5 mg/mL) was mixed with 1 mL of 1% rat liver homogenate, then 50 µL $FeCl_2$ (0.5 mM) and 50 µL H_2O_2 (0.5 mM) were added. The mixture was incubated for 60 min at 37 °C, and then 1 mL trichloroacetic acid (15%) and 1 mL 2-thiobarbituric acid (0.67%) were added. The mixture was heated in boiling water for 15 min. The experiment was performed in triplicate and the absorbance was read at 532 nm. Trolox was used as the reference product. The percentage inhibition was calculated using the following formula:

Percentage inhibition (%) = $[1\text{-}(A_1 - A_2)/A_0]$x100

A_1 is the absorbance of the control (without sample); A_2 is the absorbance with the sample; A_0 is the absorbance without liver homogenate.

2.7 Anti-Inflammatory Activity

2.7.1 Phospholipase A2 ($_s$PLA2) inhibition assay

"The sPLA2 activity of bee venom was determined according to the instructions of the manufacturer Abcam (Japan) described in catalog no. ab133089" [4, 14]. "A 96-well microplate was used to perform the sPLA2 inhibition assay. A final concentration of 100 µg/mL of HE_FDM, AM_FDM and Betamethasone (reference compound) was used. Absorbances were read spectrophotometrically (Agilent 8453) at 415 nm against a blank that had not received the enzyme. The experiment was performed in triplicate and the percentage inhibition of sPLA2 at 100 µg/mL was calculated using the following formula:

Inhibition (%) = [(AE - AI)/AE]x100.

AE: Absorbance of enzyme assay - Absorbance of blank; AI: Absorbance of inhibition assay - Absorbance of blank" [4, 14].

2.7.2 Lipoxygenase Inhibition Assay

"Lipoxygenase inhibition was determined using linoleic acid (1.25 mM) as substrate" [4, 14]. "Inhibitors (HE_FDM or AM_FDM / reference substance: Zileuton) were prepared to a final concentration of 100 µg/mL. A volume of 146.25 µL of 15-lipoxygenase solution (820.51 U/mL) was added to 3.75 µL of each inhibitor. Next, 150 µL of linoleic acid was added. A spectrophotometer (Epoch Biotek Instruments, USA) was used to measure absorbances at 234 nm against enzyme-free blanks" [4, 14]. The tests were carried out in triplicate and the percentage of lipoxygenase inhibition was calculated using the formula:

Inhibition (%) = [(AE - AI)/AE]x100

AE: Absorbance enzyme test - Absorbance blank; AI: Absorbance inhibition test - Absorbance blank.

2.8 Experimental Animals

"Female NMRI (Naval Medicinal Research Institute) mice and Wistar male rats with average weights of 28±3 g and 205±15 g, respectively from the animal house of the "Institut de Recherche en Sciences de la Santé / Centre National de la Recherche Scientifique et Technologique (IRSS/CNRST), Burkina Faso" were used. The animals were placed in an enclosure at a temperature of 21-23 °C with relative humidity between 50 and 60%, and subjected to the light/dark cycle of 12 h/12 h according to the rearing conditions of these species. Standard laboratory pellets enriched with protein (29%) and free access to water in bottles were provided to the animals" [4, 14, 16].

2.9 Spasmolytic Effect of Two Extracts of *D. mespiliformis* Fruits on Isolated Rat Duodenum

The protocol used has been described by Nitiéma et al [4]. "Wistar rat is fasted for 24 h before the start of the experiment and then sacrificed. Part of the duodenum is removed and immediately placed in Tyrode's oxygenated physiological solution [KCl (0.2 g), NaCl (8 g), $MgCl_2$ (0.01 g), NaH_2PO_4 (0.05 g), $NaHCO_3$ (1 g), $CaCl_2$ (0.2 g), and Glucose (1 g) in 1000 mL of distilled water]. A fragment (1.5 cm) was freed of adhesions and mounted in the isolated organ bath thermostated at 37 °C, with a pneumatic bubbler for organ oxygenation. One end of the isolated intestine fragment is attached to the hook of the support rod, and the other end to the isometric transducer, which in turn is connected to the recorder via an amplifier. This device visualizes the contractions of the isolated rat duodenum. The Tyrode solution is renewed every 15 min during the 45 min stabilization period". KCl (80 mM) is administered into the organ vessel to stimulate the duodenum after observing the regularity of its contractile activity, followed by rinsing. Solutions of HE_FDM and AM_FDM are administered after precontraction with Acetylcholine (ACh, 10^{-6} M) or Barium chloride ($BaCl_2$, 160 µg/mL). This makes it possible to assess the interaction of extracts with the cholinergic system and potassium fluxes in the cells. The percentage inhibition of contraction (PI) is calculated using the following formula:

PI= (h1 - h2 /h1) × 100

h1: height of peaks due to contractor alone; h2: height of peaks due to contractor in the presence of extract.

2.10 Acute Oral Toxicity

"The acute oral toxicity test for HE_FDM and AM_FDM was performed on female NMRI (Naval Medicinal Research Institute) mice by OECD guideline 423" [4, 17]. Two batches, each consisting of three mice, were made up and placed separately in polypropylene cages, a control batch of 03 mice and a test batch of 03 mice. HE_FDM or AM_FDM was administered by gavage using an esophageal tube in a single dose to the test mice after fasting for 3 h. A dose of 2000 mg/kg body weight of HE_FDM and AM_FDM was chosen as the starting dose. The control batch received the solvent for dissolving the extract (distilled water, 10 mL/kg). The mice were observed individually for 2 h after administration, at the end of which food was restored. They were then observed twice daily for a period of 14 days to monitor for mortality and signs of toxicity such as bleeding, excitement, convulsions, vomiting, salivation, hyperventilation, diarrhea, lack of appetite, sleep, and coma. The weight of each mouse and the quantities of water and food consumed were measured every 2 days during 14 days of experimentation. On day 15, the mice were sacrificed and a necropsy was performed on the organs (liver, kidneys, lungs, spleen, and heart) and then weighed. The relative weight of each organ was calculated [(Organ weight (g) / Fasting mouse weight on the day of sacrifice (g)) ×100]. This test was repeated after the mice were sacrificed under the same conditions as the 2 other batches (control batch and extract batch).

2.11 Statistical Analysis

Values are given as arithmetic means±SEM and means±SD. The concentration-response curves were constructed using GraphPad Prism 8.4.3 Software, San Diego, CA. Two pharmacological parameters were obtained: the maximal effect generated by the agonist (Emax) and a concentration of agonist inhibiting 50% of the maximum response (IC_{50}). Statistical comparisons were performed using Student's T-test, and one-way ANOVA. A post hoc test was performed using Bonferroni's test analysis to compare all the groups. A *p*-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 HPTLC Phytochemical Investigation

The phytochemical analysis of HE_FDM and AM_FDM highlighted the presence of saponosides, tannins, flavonoids, and sterol-triterpenes (Table 1).

3.2 Total Phenolics and Flavonoids Contents in Extracts of *D. mespiliformis* Immature Fruits

The total phenolics and flavonoid contents in extracts of *D. mespiliformis* immature fruits are shown in Table 2. These results are expressed in milligrams of quercetin equivalent per gram of dry extract (mg QE/g) for flavonoid and in milligrams of tannic acid equivalent per gram of dry extract for total phenolics (mg TAE/g). HE_FDM and AM_FDM showed a similar value of Total phenolics and flavonoids compound content.

3.3 Antioxidant Activity

The antioxidant activity of *D. mespiliformis* immature fruit extracts is shown in Table 3. HE_FDM and AM_FDM showed lower antioxidant activity with DPPH and ABTS than Trolox (*p<0.05). The IC_{50} for Trolox were 6.34±0.04 µg/mL and 3.78±0.21 µg/mL

Table 1. Phytochemical profile of hydroethanolic extract (HE_FDM) and aqueous macerate extract (AM_FDM) of *D. mespiliformis* immature fruits

Extracts	Saponosides	Tannins	Flavonoids	Sterol-triterpenes
HE_FDM	+	+	+	+
AM_FDM	+	+	+	-

+: presence of compound; -: compound not detected

Table 2. Total phenolics and flavonoid contents in extracts of *D. mespiliformis* immature fruits

Extracts	**Total phenolics** (mg TAE/g)	**Flavonoids** (mg QE/g)
HE_FDM	71.32±2.26	16.37±0.24
AM_FDM	61.23±0.67	15.83±0.21

QE: quercetin Equivalent; TAE: Tannic Acid Equivalent

respectively for DPPH and ABTS. The ferric ion reduction capacity (FRAP) showed similar values for both extracts. The lipid peroxidation inhibitory power (LPO), expressed as a percentage (at 100 µg/mL) of the two extracts was comparable to that of Trolox, 48.11±3.88% ($p>0.05$).

3.4 Anti-inflammatory Activity *In vitro*

The evaluation of the *in vitro* anti-inflammatory activity of the two extracts by inhibiting 15-lipoxygenase and Phospholipase A_2 is recorded in Table 4. HE_FDM, and AM_FDM have similar effects in terms of inhibition on 15-lipoxygenase. However, the Zileuton presented a better IC_{50}, 2.92±0.32 µg/mL ($p<0.001$). Phospholipase A2 activity of HE_FDM and AM_FDM showed that the extracts had a low inhibitory power compared to Betamethasone, 35.39±3.31% ($p<0.001$).

3.5 Antispasmodic Effect of Two Extracts of *D. mespiliformis* Immature Fruits on Rat Duodenum

Relaxation of isolated rat duodenum by HE_FDM and AM_FDM is shown in Fig 1. Fig 1A shows the relaxation curves for both extracts and control on the isolated duodenum precontracted with ACh. HE_FDM curve was more deviated to the left compared to AM_FDM and control. Fig 1B shows the Emax of HE_FDM (98.87±5.14%), AM_FDM (64.28±10.75%) and control (7.08±4.68%) ($p<0.05$). Fig 2A shows similar relaxation results obtained with the extracts and the vehicle (control) on the isolated rat duodenum pre-contracted with $BaCl_2$. With a statistically significant difference, the best efficacy of 81.61±7.56% was obtained with the HE_FDM (Fig 2B).

3.6 Acute Oral Toxicity

3.6.1 Animal behaviour

The dose of 2000 mg/kg body weight showed no signs of mortality or remarkable behavioral changes in female mice at the first and second stages of administration of HE_FDM, and AM_FDM (Table 5).

3.6.2 Evolution in Body Weight, Feed, and Water Consumption of Mice after 14 Days of Monitoring

Fig 3 shows the mean weight gain, feed consumption, and water consumption for 2 weeks in female mice given a vehicle (distilled water, 10 mL/kg) or a single dose (2000 mg/kg) of HE_FDM, and AM_FDM. There was no statistically significant difference between the parameters of the 03 study batches.

Table 3. *In vitro* antioxidant activity of *D. mespiliformis* immature fruits extracts

Substances	ABTS		DPPH		FRAP	LPO
	IC_{50} (µg/mL)	ARP	IC_{50} (µg/mL)	ARP	mol EAA/g	Inhibition (%) (at 100 µg/mL)
HE_FDM	6.73±0.46*	0.15	13.82± 1.04**	0.07	1149.35±13.43	41.86±5.12
AM_FDM	33.87±0.86***	0.03	83.07±6.40***	0.01	1091.95±5.12	60.75±5.09
Trolox	3.78±0.21	0.26	6.34±0.04	0.16	-----	48.11±3.88

*HE_FDM: hydroethanolic extract; AM_FDM: aqueous macerate extract; IC_{50}: inhibition concentration 50%; ARP: anti-free radical power; n = 3; *p<0.05; **p<0.01; ***p<0.001 vs Trolox; EAA: Ascorbic acid equivalent*

Table 4. 15-Lipoxygenase and phospholipase A_2 inhibitory activity of *D. mespiliformis* immature fruits extracts

Substances	15-Lipoxygenase IC_{50} (µg/mL)	Phospholipase A_2 Inhibition (%)
HE_FDM	15.11±1.39***	5.77±0.63$^{###}$
AM_FDM	19.12±2.01***	5.92±0.59$^{###}$
Zileuton	2.92±0.32	----
Betamethasone	----	35.39±3.31

*HE_FDM: Hydroethanolic Extract; AM_FDM: Aqueous Macerate Extract; ***p<0.001 vs Zileuton; ###p<0.001 vs Betamethasone; n = 3*

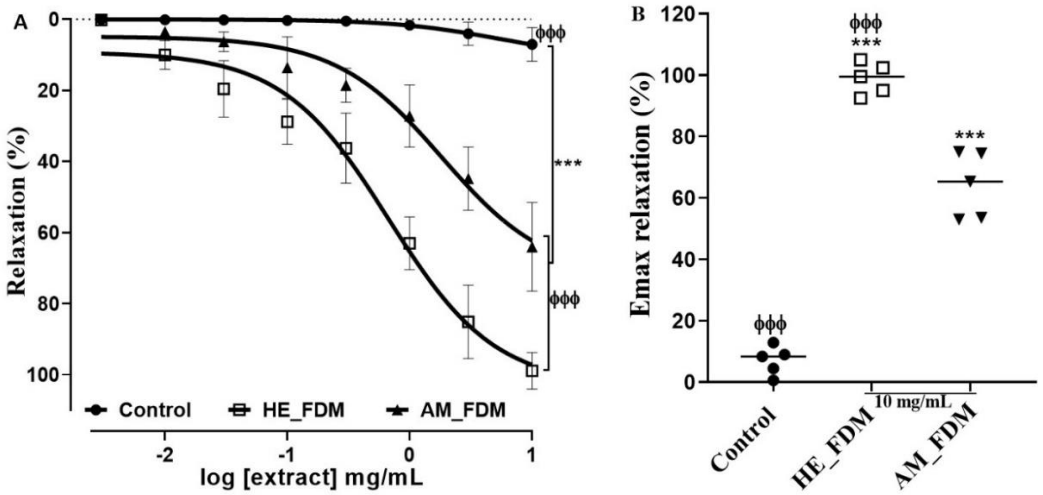

Fig. 1. Relaxation curves of Hydroethanolic extract (HE_FDM) and aqueous macerate extract (AM_FDM) of *D. mespiliformis* immature fruits on isolated ACh-precontracted rat duodenum (A) and histogram of maximum relaxation effect (B) ;

****p<0.001 vs Control; ϕϕϕp<0.001 vs AM_FDM; n = 5*

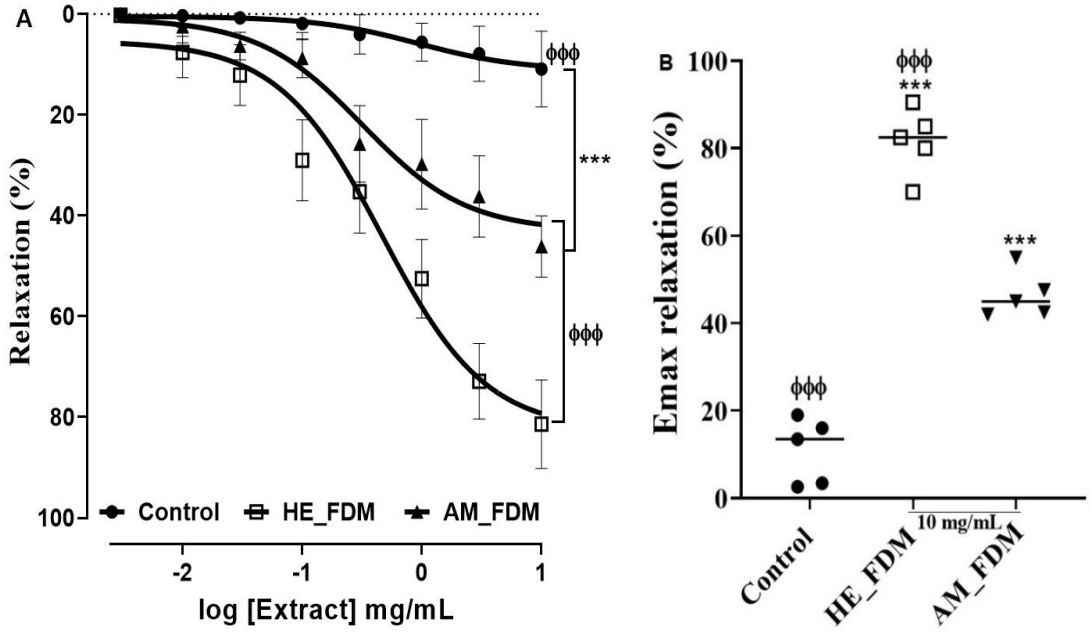

Fig. 2. Relaxation curves of Hydroethanolic extract (HE_FDM) and aqueous macerate extract (AM_FDM) of *D. mespiliformis* fruits on isolated $BaCl_2$-precontracted rat duodenum (A) and histogram of maximum relaxation effect (B)

*n = 5; ***p < 0.001 vs Control; ϕϕϕp<0.001 vs AM_FDM*

3.6.3 Macroscopic observation and relative organ weights of mice

Fresh macroscopic examination of vital organs such as the lungs, liver, heart, kidneys, and spleen of control, HE_FDM-treated, and AM_FDM-treated mice showed that there were no visually apparent changes in color or appearance or lesions of the various organs. In addition, Table 6 shows the relative organ weights of batches of control mice and mice treated with the two extracts. No statistically significant variation was observed between the relative organ weights.

Table 5. Mortality of female mice administered a single dose of *D. mespiliformis* immature fruits extracts

Extract administered	Mortality	
	1st test	2nd test
Control (distilled water)	0/3	0/3
HE_FDM (2000 mg/kg)	0/3	0/3
AM_FDM (2000 mg/kg)	0/3	0/3
Excitement	--	--
Sleepiness	--	--
Hair standing up	--	--
Lack of appetite	--	--
Diarrhea	--	--
Hyperventilation	--	--
Convulsions	--	--
Bleeding	--	--
Coma	--	--

HE_FDM: Hydroethanolic Extract; AM_FDM: Aqueous Macerate Extract

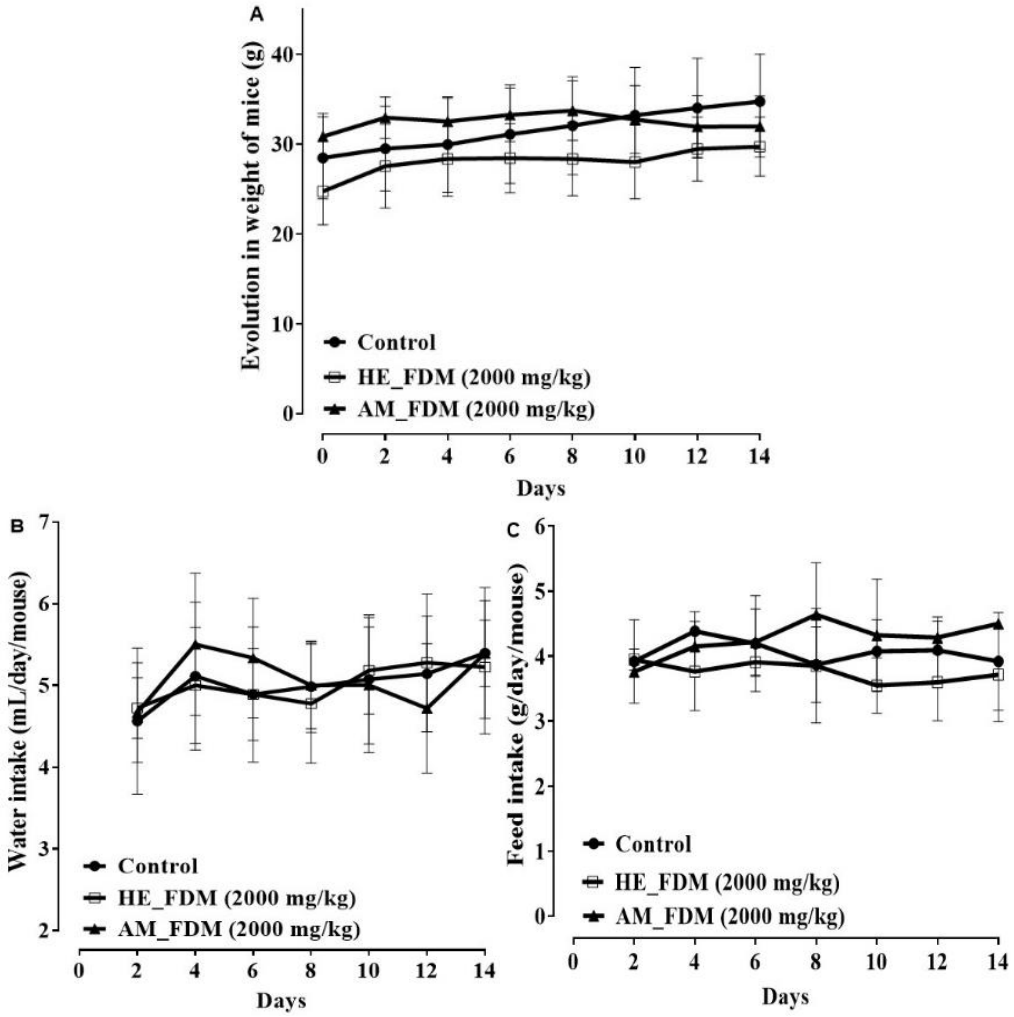

Fig. 3. Changes in weight (A), water (B) and feed (C) consumption of female mice from control and test batches with extracts of *D. mespiliformis* immature fruits during 2 weeks of follow-up

HE_FDM: Hydroethanolic Extract; AM_FDM: Aqueous Macerate Extract; n = 6

Table 6. Relative weights of female mice from control and test batches with *D. mespiliformis* immature fruits extracts during 2 weeks of follow-up; n = 6

	Relative organ weight (mean±SD)				
Substances	**Heart**	**Kidneys**	**Lungs**	**Liver**	**Spleen**
Control (Distilled water)	0.52±0.03	1.17±0.03	0.80±0.04	5.08±0.3	0.42±0.03
HE_FDM (2000 mg/kg)	0.51±0.05	1.22±0.04	0.79±0.06	5.15±0.16	0.45±0.03
AM_FDM (2000 mg/kg)	0.48±0.09	1.30±0.08	0.83±0.07	4.96±0.38	0.43±0.06

HE_FDM: Hydroethanolic Extract; AM_FDM: Aqueous Macerate Extract

4. DISCUSSION

Plants contain a multitude of bioactive compounds and as a result, have been used for thousands of years in the treatment of numerous pathologies. The treatment of gastrointestinal disorders is no exception [18]. “The leaves of *Diospyros mespiliformis* are widely used as an antispasmodic” [19]. “The phytochemical screening of the two extracts from the immature fruits of the plant is comparable to that reported in studies showing that *D. mespiliformis* immature fruits contain flavonoids, tannins, sterols, terpenes, and saponosides” [4, 20]. These compounds were also found in the plant's leaves and *Diospyros hoyleana* leaves [4, 21]. In addition, tannins had been found in immature fruit [22]. Indeed, these phytochemicals are endowed with antioxidant properties that can trap free radicals and block the implications of oxidative stress [14]. The antioxidant properties of methanolic fruit extract have been demonstrated and our results corroborate these data [23]. “In addition, according to the literature, flavonoids have anti-inflammatory and antispasmodic properties. Recent studies have shown that, like fruit extracts, the ethyl acetate fraction (84.15±1.73 mg TAE/g) and the residual aqueous fraction (89.67±2.35 mg TAE/g) of plant leaves contain total phenolics. These compounds are known for their spasmolytic properties” [4, 24, 25]. “Furthermore, the phenolics compounds, in particular tannins, flavonoids, and triterpenes present in the two extracts in the study are inhibitors of pro-inflammatory enzymes involved in the production of free radicals and the regulation or protection of the antioxidant defense system” [26, 27]. “In addition, increased spasmodic activity and diarrhea activate inflammatory pathways. Interestingly, the fruit extracts have anti-inflammatory activity albeit moderated by inhibition of phospholipase A2 and 15-lipoxygenase. These results confirm the anti-inflammatory properties of the plant, especially the leaves” [4, 28]. “Indeed, the flavonoids and sterols/triterpenes present in HE_FDM and AM_FDM are known for their ability to inhibit pro-inflammatory enzymes” [14, 29]. “The fruits of *D. mespiliformis* used in the treatment of dysentery contain tannins and saponosides which may be responsible for the effect. The tannins in the fruit of *D. mespiliformis* have anti-dysenteric effects” [13]. This evidence could justify the spasmolytic activity of the plant's fruit extracts. Pharmacological investigations showed that HE_FDM and AM_FDM have relaxing properties on isolated rat duodenum by inhibiting the stimulatory action of ACh on muscarinic receptors. Of the two extracts, HE_FDM was the more effective. ACh induces contraction by activating G protein-coupled smooth muscle M3 receptors, leading via Inositol Triphosphate (IP_3) to the release of intracellular Ca^{2+}. Its action causes a significant positive tonotropic effect marked by contractile activity reflecting the increased peristalsis of the gastrointestinal tract [30, 31]. This curative effect shows that HE_FDM and AM_FDM have anticholinergic properties. The tannins, flavonoids, saponosides and sterol-triterpenes in HE_FDM and AM_FDM, known for their anti-diarrheal and spasmolytic properties, are thought to inhibit the action of ACh [32]. Work on *D. mespiliformis* leaves fractions also showed spasmolytic effects on an isolated rat duodenum, with efficacy for the ethyl acetate fraction (Emax = 90.40±7.84%) compared with the residual aqueous fraction [4]. “In addition, HE_FDM and AM_FDM have voltages-dependent calcium channel antagonist activity through inhibition of $BaCl_2$-induced smooth muscle contraction. Indeed, both extracts at concentrations of 0.03-10 mg/mL induced a relaxing effect on the rat duodenum by significatively reducing contractions in a concentration-dependent manner. These extracts are thought to have a similar action to papaverine with a musculotropic effect, by inhibiting phosphodiesterase” [33]. “In addition, the elevated cytoplasmic Ca^{2+} concentration in smooth muscle cells is the main stimulus for duodenal contraction. This results from an influx of extracellular Ca^{2+} and/or intracellular release of stored Ca^{2+}” [34]. “Interestingly, the concentration-dependent spasmolytic effect of HE_FDM and AM_FDM on

the contractile activity of the isolated rat duodenum may be the result of Ca^{2+} uptake by phosphorylated proteins under the influence of protein kinase activated by increased levels of adenosine 3,5-cyclic monophosphate (cAMP). It may also be due to an inhibition of calcium influx or an increase in calcium efflux without altering influx" [4, 25, 33]. "For their proven spasmolytic properties, HE_FDM is the most effective even compared to the plant leaf fractions. The safety of a pharmaceutical product is a very important criterion in therapeutics. For this reason, HE_FDM and AM_FDM were evaluated for acute oral toxicity. The results showed that their LD_{50} is estimated at 5000 mg/kg bw according to the United Nations Globally Harmonised System with demonstrated adverse effects" [17]. "These results show that the immature fruits of the plant are practically non-toxic and can therefore be used appropriately. In line with the literature, studies have shown that factions and extracts from the leaves and bark of the trunk of *Diospyros mespiliformis* can be consumed without risk" [4, 35]. "Also, the aqueous extract of the leaves of *Diospyros hoyleana* F.white (Ebenaceae) has an LD_{50} greater than 5000 mg/kg" [21]. However, the mechanism of action of these fractions requires further investigation, in particular the bio-guided evaluation of their *in vivo* anti-diarrheal properties and their medium- and long-term oral toxicity.

5. CONCLUSION

This study provides evidence that *Diospyros mespiliformis* immature fruits of can be used safely to treat intestinal spasms and diarrhea. This finding is the first to our knowledge to pharmacologically demonstrate the spasmolytic effects of the immature fruits of this plant. The results showed that hydroethanolic and aqueous extracts of *D. mespiliformis* immature fruits inhibit ACh- and $BaCl_2$-induced contractions of the intestinal muscle. Tannins, saponosides, flavonoids, sterols, and terpenoids are thought to be responsible for the antioxidant, anti-inflammatory, and spasmolytic properties. The present study would therefore justify the traditional use of this plant for gastrointestinal symptoms. However, there is still a need to understand the mechanisms involved and to ensure that extracts are safe in the medium and long term.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Experiments were conducted by the procedures of the Helsinki Declaration Guide to Good Practice in Animal Experimentation. In addition, experimental procedures on mice and rats were carried out by the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and with Directive 2010/63/EU on animal experimentation.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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