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Research Article

Phytochemical profile, acute oral toxicity, antioxidant, and antispasmodic effects of ethyl acetate and aqueous residual fractions of *Diospyros mespiliformis* Hochst. ex A. DC (Ebenaceae) leaves on isolated duodenum of rat

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Abstract

Introduction: *Diospyros mespiliformis* Hochst. ex A. Rich (Ebenaceae) is a nutritional, artisanal and medicinal plant. It is used in alternative medicine in Burkina Faso for the treatment of conjunctivitis, menorrhagia, dysentery, and especially diarrhea. **Aims:** Our study aimed to evaluate the chemical profile, the antioxidant and anti-inflammatory activities, the safety of use, and the spasmolytic effects of the fractions obtained from the aqueous decoction of the leaves of *Diospyros mespiliformis.* **Methods:** Phytochemical screening by HPTLC and the determination of compounds of interest were carried out. The antioxidant activity was evaluated according to the ABTS, DPPH, FRAP, and LPO methods. The inhibitory activity of Phospholipase A2 and 15-lipoxygenase was evaluated. Acute oral toxicity was carried out on female mice (NMRI). The *ex vivo* spasmolytic effect of the fractions was tested on isolated rat duodenum using ACh and $BaCl_2$ as contracting agents. **Results:** At the end of these tests, the fractions contain flavonoids, tannins, sterols, triterpenes, and saponosides. The content of total phenolics was respectively for the ethyl acetate fraction (EAF) and the residual aqueous fraction (RAF) 84.15±1.73 mg EAT/g and 89.67±2.35 mg EAT/g. That of flavonoids was respectively 45.91 ± 0.98 mg EQ/g and 10.46±0.28 mg EQ/g for the two fractions. The 50% inhibitory concentrations (IC_{50}) of EAF for the ABTS, DPPH, and FRAP tests were 2.26±0.16 µg/mL, 22.34±7.23 µg/mL and 1136.25±0.90 mol EAA/g respectively, and an inhibition % of 43.80 ± 6.31% for LPO. For RAF, the IC_{50} were in the same range as EAF of 16.82±0.23 µg/mL, 18.58±2.91 µg/mL, 1138.4±1.27 mol EAA/g and an inhibition of 45.14±10.35% for LPO. The fractions had an inhibitory effect on phospholipase A2 compared to Betamethasone. Finally, the most active EAF caused a spasmolytic effect with Emax of 87.4±15.71% and 90.40±7.84%, respectively, during contractions induced by $BaCl_2$ and ACh. **Conclusion:** Finally, this work provided scientific data and could justify the use of *D. mespiliformis* leaves in the treatment of diarrhea.

Keywords: *Diospyros mespiliformis*, Leaves, Antioxidants, Anti-inflammatory, Safety of use, Spasmolytic

INTRODUCTION

In alternative medicine, plants are a source of bioactive compounds. In fact, 80% of the population in developing countries rely on these bioactive compounds for their healthcare [1]. Gastrointestinal diseases also figure prominently among the population's various ailments. In 2019, there were 8 million deaths from digestive diseases, and this figure is not in decline over three decades [2]. The spasmolytic effect of drugs is commonly used to reduce excessive contractility of the intestine, responsible for cramps and discomfort in the abdominal region [3]. Several strategies are used in this treatment, both in modern and traditional medicine, using medicinal plants. In scientific terms, ethnobotanical, biochemical, pharmacological, and toxicological studies and clinical trials have been conducted to provide evidence of the use of plant drugs [4-6]. To this end, among the plant species widely used for their medicinal properties is *Diospyros mespiliformis* Hochst. ex A. DC (Ebenaceae) [6]. Studies carried out on this plant have demonstrated the anti-proliferative properties of trunk bark extracts [7] and the antioxidant and antimicrobial activities of organic leaf extracts [6, 8]. The anti-

plasmodial activity of *Diospyros mespiliformis* trunk bark and leaf extracts on *Plasmodium berghei* has also been demonstrated [6, 9]. The inhibition of the α-glucosidase enzyme by bioactive compounds isolated from *Diospyros mespiliformis* has been documented [10]. Preliminary studies have shown that the aqueous decoction of the plant's leaves has spasmolytic effects. However, the antispasmodic properties of leaf fractions have not yet been elucidated. It was therefore essential to assess the safety and spasmolytic efficacy of the ethyl acetate and aqueous residual fractions of *Diospyros mespiliformis* leaves.

MATERIAL AND METHODS

Chemicals and Reagents

Chloroform, Ethyl acetate, Methanol, formic acid, Hexane, Dimethyl sulfoxide (DMSO), NEU reagent, aluminum trichloride, iron chloride, ferric trichloride, Folin Ciocalteu reagent (FCR), sulphuric anysaldehyde reagent, Liebermann and Burchard reagent, monobasic potassium phosphate, sodium phosphate dibasic, 15-lipoxygenase (EC 1.13.11.12), linoleic acid, sodium bicarbonate, potassium hexacyanoferrate, trichloroacetic acid (TCA), thiobarbituric acid (TBA), hydrogen peroxide solution, 2,2'-azino bis-[3-éthylbenzothiazoline-6-sulfonique] (ABTS), 2,2-diphenyl-β-picrylhydrazyl (DPPH), and potassium persulfate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gallic acid, Quercetin, Ascorbic acid, Trolox, Betamethasone, and Zileuton were supplied by Sigma Aldrich. Silica gel TLC plates F 254 grade was from Macherey-Nagel (Germany),

Plant material

Fresh leaves of *Diospyros mespiliformis* Hochst. ex A. DC (Ebenaceae) were collected in May 2022 in Goundi, in the Sanguié Province of Burkina Faso. A botanist from the Laboratoire de biologie végétale et d'écologie de l'Université Joseph KI-ZERBO, Burkina Faso, identified and authenticated a sample. A specimen exists under number 4267 (OUA). The leaves were dried in the shade, away from direct sunlight and with ventilation. After drying, the leaves were pulverised using a mechanical grinder (Gladiator Est. 1931 Type BN 1 Mach. 404611083) to obtain a dry extractive powder.

Preparation of the leaves fractions

Leaves fractions were prepared by making an aqueous *Diospyros mespiliformis* leaves powder decoction. One hundred (100) g of the plant powder was dissolved in 500 mL of distilled water and boiled for 30 min. After cooling, the supernatant was filtered through a fine mesh nylon cloth and centrifuged at 2000 rpm for 5 min to give the aqueous decoctate. The decoctate was subjected to a liquid-liquid fractionation with ethyl acetate. This organic phase was concentrated in a rotavapor at 70 °C and oven-dried at 60 °C to give the ethyl acetate fraction, EAF (0.85%). The residual aqueous phase was freeze-dried to give the residual aqueous fraction, RAF (11.33%). These 2 fractions were used for the various tests.

Phytochemical Investigation: High-performance thin-layer chromatography

High-performance thin-layer chromatography (HPTLC) was used to detect flavonoids and tannins in the two fractions (EAF and RAF). It was carried out on chromatoplates (60 F_{254}, 10 x 5 cm, glass support 10 x 20 cm, Merck) following the literature [11]. 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 (2 × 1 dm, saturation time: 30 min). The solvent system used depended on the metabolite to be identified: ethyl acetate/formic acid/H_2O, (8/2/1 v/v/v/) for flavonoids; ethyl acetate/formic acid/H_2O (18/2/4/2/1 v/v/v/v) for tannins; 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 8 cm in length, the plates were dried, and Neu reagent for flavonoids, sulphuric anisaldehyde reagent for saponosides, 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) and at UV wavelengths of 366 nm.

Determination of Flavonoid Compound

Flavonoid content was assessed using an Aluminium chloride reagent [12]. A standard calibration curve was plotted with Quercetin. one hundred (100) µL of EAF or RAF (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.

Assessment of Antioxidant Properties

DPPH• Essay

Free radical scavenging activity of EAF, RAF, and Trolox was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as previously described [13]. 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. 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

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed on EAF, RAF and Trolox as previously described [13]. 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 EAF and RAF was expressed as mol Trolox equivalent/gram dry extract.

ABTS•+ Assay

The ABTS free radical scavenging activity of EAF, RAF and Trolox was assessed using the procedure described previously [12]. 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 EAF or

RAF 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. The percentage inhibition of absorbance at 415 nm was calculated according to the formula:

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

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

Lipid Peroxidation Inhibition (LPO) Assay

The lipid peroxidation activity of rat liver was determined using 2-thiobarbituric acid (Sigma Aldrich) [12]. $FeCl_2$-H_2O_2 was used to induce peroxidation of liver homogenate. A 0.2 mL volume of EAF or RAF (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, 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 (%) = $[1-(A_1 - A_2)/A_0]x100$

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.

Anti-Inflammatory Activity

Phospholipase A2 (sPLA2) Inhibition Assay

The sPLA2 activity of bee venom was determined according to the instructions of the manufacturer Abcam (Japan) described in catalog no. ab133089 [12]. A 96-well microplate was used to perform the sPLA2 inhibition assay. A final concentration of 100 µg/mL of EAF, RAF 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.

Lipoxygenase Inhibition Assay

Lipoxygenase inhibition was determined using linoleic acid (1.25 mM) as substrate [12]. Inhibitors (EAF or RAF / reference substance: Zileuton) were prepared to a final concentration of 100 µg/mL. 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. 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.

Experimental animals

Female NMRI (Naval Medicinal Research Institute) mice and Wistar male rats with average weights of 27 ± 4 g and 185 ± 23 g, respectively from the animal house of the "Institut de Recherche en Sciences de la Santé/Centre National de 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 a relative humidity of 55 ± 5% 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 proteins (29%) and water were provided for satiation and experiments were carried out following the procedures of the Guide of Good Practices in Animal Experimentation under the Declaration of Helsinki. Furthermore, all experimental animal procedures have been performed by the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and the EU Directive 2010/63/EU for animal experiments [12, 13].

Acute Oral Toxicity

The acute oral toxicity test for EAF and RAF was performed on female NMRI (Naval Medicinal Research Institute) mice in accordance with OECD guideline 423[15]. 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. EAF and RAF 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 EAF and RAF was chosen as the starting dose. 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 hair standing up, excitement, vomiting, hyperventilation, diarrhea, lack of appetite, sleepiness. 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) 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).

Spasmolytic effect of two fractions on isolated rat duodenum

The protocol used has been described by Boly et al. [14]. Wistar rat is fasted for 24 h before the start of the experiment and then sacrificed. A portion 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), $NaHCO_3$ (1 g), NaH_2PO_4 (0.05 g), $CaCl_2$ (0.2 g), and Glucose (1 g) in 1 L of distilled water]. A 15 mm fragment 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. After observing the regularity of the contractile activity of the isolated organ, KCl (80 mM) is administered into the vessel to stimulate the organ, followed by rinsing. Solutions of the fractions (EAF and RAF) are administered on the one hand, and on the other, after precontraction with acetylcholine (ACh, 10^{-6} M) or barium chloride ($BaCl_2$, 160 µg/mL). This makes it possible to assess, respectively, the extract's effect on normal contractile activity

in the isolated intestine and its interaction with the cholinergic system and potassium fluxes in the cells. The percentage inhibition of contraction (PI) is calculated using the following formula:

$$PI = (h1 - h2 / h1) \times 100$$

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

Statistical analysis

Values are given as arithmetic means ± SEM. The significance of differences between means was conducted by GraphPad Prism in version 8.4.3. Student's t-test, one- and two-way ANOVA, followed by Bonferroni multiple comparison tests were used for comparisons. The difference was considered statistically significant for a threshold of *p*-value < 0.05.

RESULTS

HPTLC phytochemical investigation

The phytochemical analysis of EAF and RAF highlighted the presence of saponosides, tannins, flavonoids, and sterol-triterpenes (Figure 1).

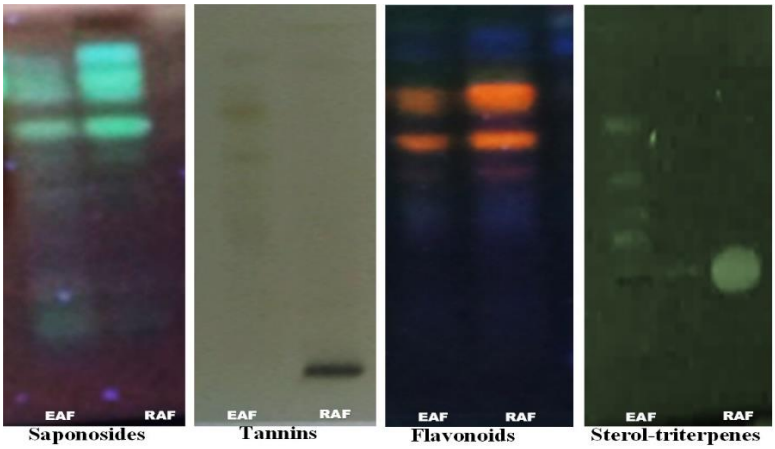

Figure 1: Phytochemical profile of ethyl acetate fraction (EAF), and residual aqueous fraction (RAF) revealed by HPTLC

Total phenolic and flavonoid contents in *D. mespiliformis* fractions

Table I: Total phenolic and flavonoid content of ethyl acetate and residual aqueous fractions

Extracts	Total phenolics (mg TAE/g)	Flavonoids (mg QE/g)
EAF	84.15 ± 1.73	45.91 ± 0.98
RAF	89.67 ± 2.35	10.46 ± 0.28***

*QE: quercetin equivalent; TAE: tannic acid equivalent; ***p < 0.001 vs EAF*

The total phenolic and flavonoid contents are shown in Table I. These results are expressed in milligrams of tannic acid equivalent per gram of dry extract for total phenolics (mg TAE/g) and in milligrams of quercetin equivalent per gram of dry extract (mg QE/g) for flavonoids. The two fractions showed the similar value of Total phenolic compound content and Ethyl acetate fraction had a high flavonoid compound content.

Biological activities

Antioxidant activity

The antioxidant activity of *Diospyros mespiliformis* leaves fractions is shown in Table II. EAF had an IC_{50} of 2.26±0.16 µg/mL using the ABTS test. This was statistically significant compared to Trolox (3.78±0.21 µg/mL). For the DPPH radical reduction method, the IC_{50} were 22.34±7.23 µg/mL and 18.58±2.91 µg/mL respectively for EAF and RAF. Significance was obtained between the IC_{50} of these two fractions and the reference compound (Trolox, 6.34±0.04 µg/mL). The ferric ion reduction capacity (FRAP) was 1136.25±0.90 mol EAA/g (EAF) and 1138.4±1.27 mol EAA/g (RAF). The lipid peroxidation inhibitory power (LPO), expressed as a percentage (%) (at 100 µg/mL) was 43.80±6.31% for the EAF, 45.14±10.35% for RAF and 48.11±3.88% for Trolox.

Table II: *In vitro* antioxidant activity of *Diospyros mespiliformis* leaves fractions

Extraits	ABTS		DPPH		FRAP	LPO
	IC_{50} (µg/mL)	ARP	IC_{50} (µg/mL)	ARP	mol EAA/g	Inhibition (%) (at 100 µg/mL)
EAF	2.26±0.16*	0.44	22.34±7.23**	0.045	1136.25±0.90	43.80±6.31
RAF	16.82±0.23***	0.059	18.58±2.91*	0.054	1138.4±1.27	45.14±10.35
Trolox	3.78±0.21	0.26	6.34±0.04	0.16	-----	48.11±3.88

EAF: ethyl acetate fraction; RAF: Residual aqueous fraction; IC_{50}: inhibition concentration 50%; ARP: anti-free radical power; n = 3; *p < 0.05; ***p < 0.001 vs Trolox for ABTS, DPPH, and LPO; EAA: Ascorbic acid equivalent.

In Vitro Anti-inflammatory activity

The evaluation of the *in vitro* anti-inflammatory activity of the two leaves fractions by inhibiting 15-lipoxygenase and Phospholipase A_2 is recorded in Table III. EAF, and RAF have similar effects in terms of inhibition on 15-lipoxygenase. However, the Zileuton presented a better IC_{50}, 2.92±0.32 µg/mL (***$p < 0.001$). The evaluation of the effect of fractions on the activity of Phospholipase A_2 expressed as a percentage of inhibition shows that there was no statistical difference between EAF, RAF, and Betamethasone (reference substance).

Table III: 15-Lipoxygenase and Phospholipase A_2 inhibitory activity of *Diospyros mespiliformis* leaves fractions

Fractions	15-Lipoxygenase IC_{50} (µg/mL)	Phospholipase A_2 Inhibition (%)
EAF	13.40±1.02***	37.53±1.92
RAF	13.08±1.46***	30.94±4.90
Zileuton	2.92±0.32	----
Betamethasone	----	35.39±3.31

n = 3 ; ***$p < 0.001$ vs Zileuton

Acute oral toxicity

The dose of 2000 mg/kg body weight (bw) showed no signs of mortality or remarkable behavioral changes in female mice at the first and second stages of administration of the residual aqueous fraction. As for EAF, the dose of 300 mg/kg did not cause mortality during the two-administration series (Table IV).

Table IV: Mortality of female mice administered a single dose of fractions from *D. mespiliformis* leaves

Fractions administered	Mortality	
	1st test	2nd test
Control (1% Tween 80)	0/3	0/3
EAF (2000 mg/kg)	3/3	--
EAF (300 mg/kg)	0/3	0/3
RAF (2000 mg/kg)	0/3	0/3
Excitement	--	--
Sleepiness	--	--
Hair standing up	--	--
Lack of appetite	--	--
Diarrhea	--	--
Vomiting	--	--
Hyperventilation	--	--

Changes in body weight, food, and water consumption of mice after 14 days of monitoring

Figure 2 shows the mean weight gain, feed consumption (g/g of mice), and water consumption (mL/g of mice) for 14 days in female mice given a vehicle (1% Tween 80, 10 mL/kg), a single dose (2000 mg/kg) of RAF, and 300 mg/kg of EAF. There was no statistically significant difference in body weight gain between the treated and control batches.

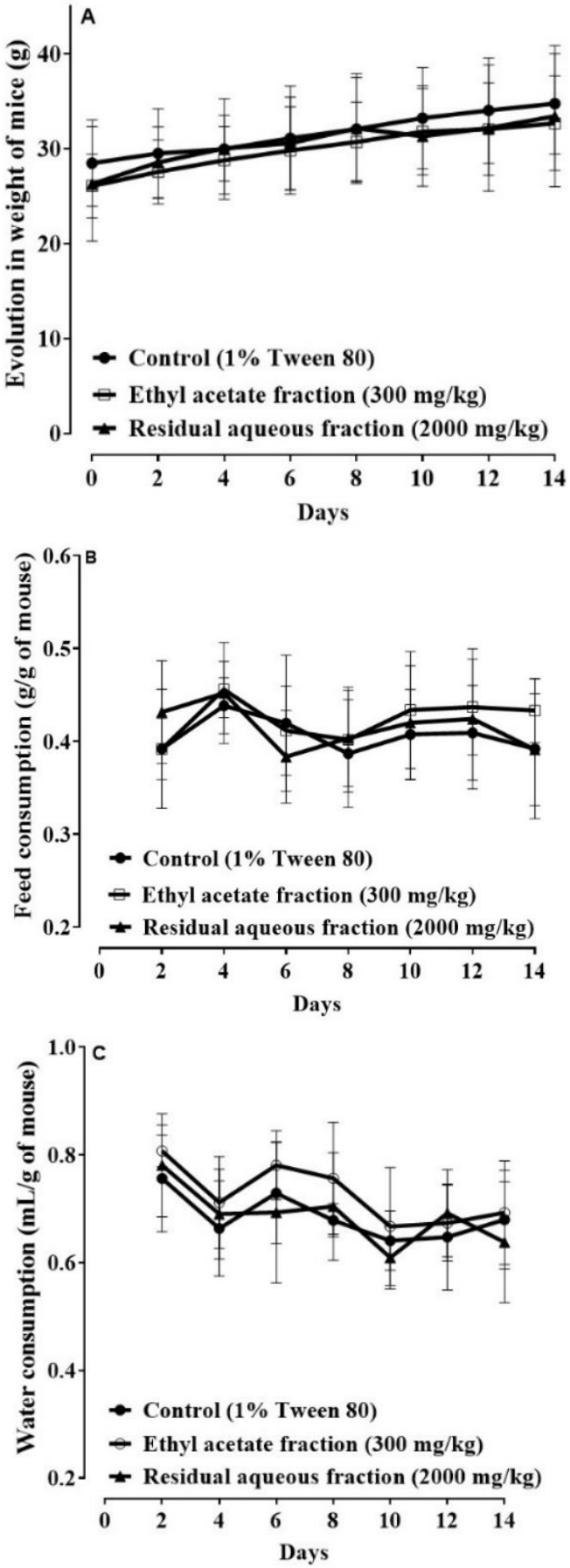

Figure 2: Changes in weight (A) and feed (B) and water (C) consumption of female mice from control and test batches with *D. mespiliformis* leaves fractions during 14 days of follow-up; n = 6

Macroscopic observation and relative organ weights of mice

Fresh macroscopic examination of vital organs such as the heart, lungs, liver, kidneys, and spleen of control mice and mice treated with EAF and RAF showed that there were no lesions, nor any change in color or appearance of the various organs. Table V shows the relative organ weights of batches of control mice and mice treated with EAF (300 mg/kg) or RAF (2000 mg/kg). No statistically significant variation was observed between the relative organ weights of control and treated batches.

Table V: Relative weights of female mice from control and test batches with *D. mespiliformis* leaves fractions during 14 days of follow-up; n = 6

Substances	Relative organ weight (mean±SD)				
	Heart	Kidneys	Lungs	Liver	Spleen
Control (Tween 80, 1%)	0.52±0.03	1.17±0.03	0.80±0.04	5.08±0.3	0.42±0.03
EAF (300 mg/kg)	0.51±0.02	1.20±0.05	0.83±0.03	5.08±0.2	0.44±0.03
RAF (2000 mg/kg)	0.53±0.06	1.22±0.04	0.79±0.06	5.15±0.16	0.45±0.03

EAF : Ethyl acetate fraction ; RAF : Residual aqueous fraction

Antispasmodic effect of EAF and RAF of *D. mespiliformis* leaves on isolated rat duodenum

The results of the relaxation of the isolated rat duodenum the two fractions (EAF and RAF) of *D. mespiliformis* leaves and vehicle are presented in Figure 3. Figure 3A shows the relaxation curves for both fractions and the control on the isolated duodenum precontracted with ACh. The EAF curve was more deviated to the left compared to RAF and control. The histogram (figure 3B) shows the Emax of EAF (90.40±7.84%), RAF (40.21±12.79%) and control (0.88±0.42%). A statistically significant difference was noted between the effects of the two fractions compared with the control. Figure 4A shows similar relaxation results obtained with the fractions and the vehicle (control) on the isolated rat duodenum pre-contracted with $BaCl_2$. With a statistically significant difference, Figure 4B presented the Emax of the EAF (87.4±15.71%) and RAF (51.81±12.88%) fractions and the control (10.91±7.48%).

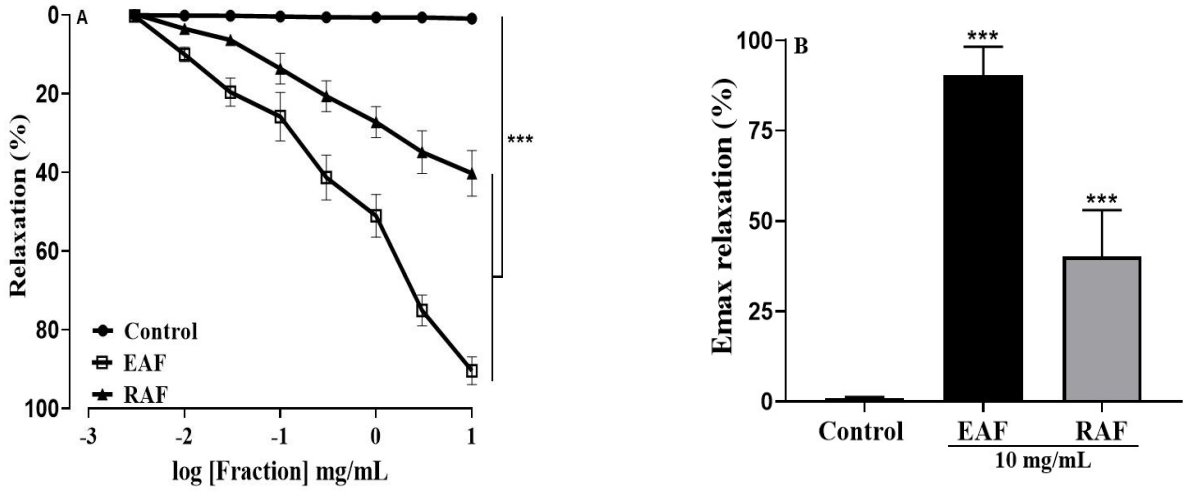

Figure 3: Relaxation curves for ethyl acetate (EAF) and residual aqueous (RAF) fractions of *D. mespiliformis* leaves on isolated ACh-precontracted rat duodenum (A) and histogram of maximum relaxation effect (B) ; n = 5 ; ***p < 0.001 vs Control

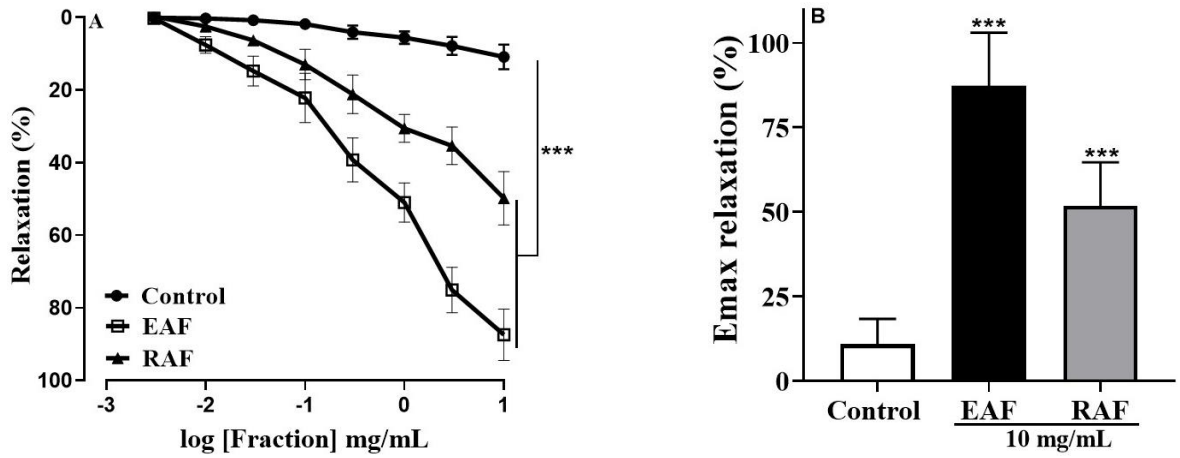

Figure 4: Relaxation curves for ethyl acetate (EAF) and residual aqueous (RAF) fractions of *D. mespiliformis* leaves on isolated $BaCl_2$-precontracted rat duodenum (A) and histogram of maximum relaxation effect (B) ; n = 5 ; ***p < 0.001 vs Control

DISCUSSION

Traditional herbal medicine has been used to treat illnesses since ancient times. This practice is used to treat gastrointestinal disorders [15]. As such, the leaves of *Diospyros mespiliformis* are widely used as an antispasmodic [16]. The aim of this study was to provide scientific data on the use of the plant in the treatment of diarrhoea in alternative medicine. The phytochemical screening of *Diospyros mespiliformis* leaves fractions is comparable to that reported in studies showing that *D. mespiliformis* leaves contain flavonoids, tannins, sterols, terpenes and saponosides [6, 17]. These phytochemicals neutralise reactive oxygen species and superoxides, while other flavonoids can trap the highly reactive oxygen radical known as peroxynitrite [13]. Flavonoids have anti-inflammatory and antispasmodic properties. They also have anti-inflammatory, anti-diarrhoeal, anti-parasitic and antibacterial properties [16] In addition, total phenolic content was measured in the ethyl acetate fraction (84.15±1.73 mg TAE/g) and the residual aqueous fraction (89.67±2.35 mg TAE/g). Higher levels of total phenolic compounds in aqueous, ethanolic, methanolic and petroleum ether extracts of *Diospyros mespiliformis* leaves have been demonstrated [6, 18]. These compounds are known for their spasmolytic properties [19]. In addition, phenolic compounds, in particular flavonoids, tannins and triterpenes, are inhibitors of certain pro-inflammatory enzymes, chelators of heavy metals involved in the production of free radicals and the regulation or protection of the antioxidant defense system [20, 21]. The antioxidant properties of *D. mespiliformis* leaves extracts have already been demonstrated [6, 22, 23]. Moreover, in the spasmodic mechanism, inflammation of the viscera cannot be ignored, hence the search for anti-inflammatory properties in the fractions. Overall, the fractions showed good inhibition of phospholipase A2 and 15-lipoxygenase. These anti-inflammatory activities were less effective than Zileuton, but similar to Betamethasone. These results confirm the anti-inflammatory properties of the plant's leaf fractions [23]. The flavonoids and sterols/triterpenes in the fractions are known for their ability to inhibit pro-inflammatory enzymes [13, 24]. In addition, for the safe use of both fractions, acute oral toxicity was assessed. The results showed that acute oral administration of the EAF fraction (2000 mg/kg bw) resulted in mortality in mice. This finding indicates that EAF should be used sparingly. However, EAF (300 mg/kg bw) and RAF (2000 mg/kg bw) did not cause any mortality or behavioural changes. Thus, the LD_{50} of these two fractions was estimated at 1000 mg/kg and 5000 mg/kg bw respectively for EAF and RAF according to the United Nations Globally Harmonised System [25]. These results suggest that at very high doses, AEF can have harmful effects on consumers. In line with the literature, work has shown that the methanolic extract of the leaves and bark of the trunk of *Diospyros mespiliformis*, as well as their hexane, ethyl acetate and butanol fractions, can be safely consumed [26]. Pharmacological results showed that EAF and RAF have muscle relaxant properties on isolated rat duodenum after stimulation of muscarinic receptors by ACh with best efficacy for EAF. ACh induces a significant positive tonotropic effect marked by a contracture with plateau contractile activity reflecting the increased peristalsis of the gastrointestinal tract. ACh induces contraction through the activation of G protein-coupled smooth muscle M_3 receptors, leading via Inositol Triphosphate (IP_3) to the release of intracellular Ca^{2+} [27, 28]. This curative experiment shows that EAF and RAF have anticholinergic properties. This property can be explained by the presence of tannins, flavonoids, saponosides and terpenoids in the fractions, which block the action of ACh. Indeed, these compounds are known for their anti-diarrhoeal properties through their spasmolytic effects [19, 29]. Moreover, these results are in agreement with the literature, which has documented the anti-diarrhoeal effects of a decoction of *D. mespiliformis* leaves, traditionally used in Ghana and Nigeria [6]. Preliminary results with the freeze-dried aqueous decoctate of the plant's leaves not documented in the present work also showed spasmolytic effects on isolated rat duodenum. More effective than EAF but weaker than RAF. The cytoplasmic increase in Ca^{2+} concentration in smooth muscle cells is the main stimulus for contraction, which usually results from both intracellular release of stored Ca^{2+} and influx of extracellular Ca^{2+} [30]. The concentration-dependent spasmolytic effect of EAF and RAF on contractile activity in the isolated gut could be the result of Ca^{2+} uptake by phosphorylated proteins under the influence of cAMP-activated protein kinase. It may also be due to an inhibition of calcium influx or an increase in calcium efflux without altering influx, causing gastrointestinal smooth muscle relaxation [19, 31]. In addition, EAF and RAF inhibit $BaCl_2$-induced smooth muscle contraction. Indeed, both fractions at concentrations of 0.03-10 mg/mL induced a relaxant effect on the rat duodenum by significatively and concentration-dependently reducing contractions. This effect could be due to an action similar to papaverine with a musculotropic effect, by inhibiting phosphodiesterase function [14]. However, the mechanism of action of these fractions needs to be studied in greater depth, especially the assessment of their *in vivo* anti-diarrhoeal properties and their medium- and long-term oral toxicity.

CONCLUSION

We are not aware of any work on the spasmolytic properties of *D. mespiliformis* leaves fractions. Results have shown that ethyl acetate and residual aqueous fractions of *D. mespiliformis* leaves inhibit the contractile actions of intestinal muscle by ACh and $BaCl_2$. These effects are thought to be mediated by tannins, saponosides, flavonoids, sterols and terpenoids, which also have antioxidant and anti-inflammatory effects. The ethyl acetate fraction was moderately toxic at high doses compared to the residual aqueous fraction. Thus, the present study contributes to new knowledge of the spasmolytic effects of *D. mespiliformis* leaves on the isolated rat duodenum, and reinforces the traditional use of this plant for gastrointestinal symptoms. However, further research is needed to improve our understanding of the mechanisms involved.

CONFLICT OF INTERESTS

Authors have declared that no competing interests exist.

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CONFLICT OF INTERESTS

Authors have declared that no competing interests exist.

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