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Phytochemical Profile, Antioxidant, and Anti-Inflammatory Activities, Safety of Use and Spasmolytic Effects of Aqueous Decoction Extract of *Diospyros mespiliformis* Leaves Hochst. ex A. DC. (Ebenaceae) on the Isolated Duodenum of Rat

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Abstract

Diospyros mespiliformis Hochst. ex A. DC. (Ebenaceae) is a multi-use plant, including for therapeutic purposes. It is used in alternative medicine in Burkina Faso to treat conjunctivitis, menorrhagia, dysentery, and diarrhea. The aim of our study was to evaluate the chemical profile, antioxidant and anti-inflammatory activities, safety of use and spasmolytic effects of the aqueous decoction of *Diospyros mespiliformis* leaves. Phytochemical screening by HPTLC and assay of compounds of interest were carried out. Four methods were used to assess antioxidant activity. Inhibitory activity against 15-lipoxygenase and phospholipase A2 was assessed. Acute oral toxicity of the extract was tested on female mice (NMRI). Following these tests, the extract contained bioactive compounds of interest such as flavonoids, tannins, sterols, triterpenes, and sa-

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ponosides. The total phenolic and flavonoid contents of the aqueous decoctate were 70.59 ± 3.20 mg EAT/g and 31.57 ± 0.78 mg EQ/g respectively. The extract was less active than Trolox with inhibitory concentrations of 50% (IC_{50}) for the ABTS, DPPH, FRAP, and LPO tests of 7.53 ± 0.08 µg/mL, 29.47 ± 0.06 µg/mL, 1128.83 ± 4.82 mol EAA/g, and 32.30 ± 1.60 µg/mL respectively. The extract has an anti-inflammatory effect with inhibition of phospholipase A2 compared to betamethasone. In addition, the aqueous extract produced an antispasmodic effect with Emax of 70% and 80% respectively during contractions induced by $BaCl_2$ and ACh. Finally, this study provided basic scientific data and could justify the use of *D. mespiliformis* leaves in the treatment of diarrhea.

Keywords

Diospyros mespiliformis, Phytoconstituents, Antioxidants, Acute Oral Toxicity, Spasmolytic, Leaves

1. Introduction

Traditional medicine remains the first line of health care for 80% of the population in developing countries [1]. In alternative medicine, plants are the source of bioactive compounds. On the scientific front, much work has been carried out on plants, including ethnobotanical, biochemical, pharmacological, toxicological studies, and clinical trials, to strengthen this cultural heritage and provide scientific evidence [2] [3] [4]. These plants contain various secondary metabolites with therapeutic properties, including anti-diarrhoeal properties. According to science, the peripheral parts of plants (leaves, barks) are where secondary metabolites or basic materials that protect the body are stored [5]. Traditional healers use these plants to treat diarrhea, often resulting from intestinal spasms. Antispasmodics or spasmolytics are medicines used to treat muscular spasms, which are intense, sudden pathological contractions of involuntary or smooth muscles. The most common spasms are those in hollow organs and sphincters: the digestive tract, urinary tract, uterus, and respiratory system [6].

In addition, several strategies are used in this treatment, both in modern and traditional medicine, using medicinal plants. Moreover, modern medicines are often very expensive and difficult to obtain. This is one of the reasons why patients in low-income countries turn to traditional plant-based remedies, which have a cultural dimension and are made up of several bioactive compounds [7]. Given this situation, developing medicinal plants by stepping up research is essential. Among the plant species widely used for their medicinal properties is *Diospyros mespiliformis* Hochst. ex A. DC (Ebenaceae) [4].

Various studies have been carried out on the plant. Thus, we have the anti-proliferative properties of *Diospyros mespiliformis* stem barks extracts [8], the antioxidant activities of methanolic, ethanolic, and petroleum ether extracts of *Diospyros mespiliformis* leaves [4]. Also, the antimicrobial activity of aqueous and ethanolic extracts of *Diospyros mespiliformis* leaves, stem bark and root

have been documented [4] [9]. The anti-plasmodial activity of the aqueous, ethyl acetate and n-butanol fractions of the methanolic extracts of *Diospyros mespiliformis* stem barks, and leaves were also tested on mice infected with *Plasmodium berghei* [4] [10].

Mohammed *et al.* tested the α-glucosidase enzyme inhibition activity of bioactive compounds isolated from *Diospyros mespiliformis* [11]. However, the anti-diarrhoeal properties of the leaves have yet to be investigated pharmacologically. It was, therefore essential to assess the spasmolytic efficacy and safety of aqueous extract from *Diospyros mespiliformis* leaves to ensure that they are used more widely by the general public.

2. Material and Methods

2.1. Chemicals and Reagents

Chloroform, Methanol, Ethyl acetate, formic acid, Hexane, Dimethyl sulfoxide, NEU reagent, aluminum trichloride, iron chloride, ferric trichloride, Folin Ciocalteu reagent (FCR), sulphuric anysaldehyde reagent, sodium phosphate dibasic, monobasic potassium phosphate, 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). Ascorbic acid, Gallic acid, Quercetin, Trolox, Betamethasone, and Zileuton were supplied by Sigma Aldrich. Silica gel TLC plates F 254 grade was from Macherey-Nagel (Germany).

2.2. Plant Material

The fresh leaves of *Diospyros mespiliformis* Hochst. ex A. DC. (Ebenaceae) were harvested in May 2022 in Goundi, province of Sanguié, Burkina Faso. A botanist Plant Biology and Ecology Laboratory, University of Joseph KI-ZERBO, Burkina Faso identified and authenticated a sample (Specimen N˚4267). The leaves were dried in the shade, out of direct sunlight, and with ventilation. After drying, the leaves were pulverized with a mechanical grinder to obtain a dry extractive powder.

2.3. Preparation of the Plant Extract

The extract was prepared by making an aqueous decoction of *Diospyros mespiliformis* leaves powder. Two hundred (200) g of the plant powder was dissolved in 1000 mL of distilled water and boiled for 30 min. After cooling, the supernatant was filtered through fine mesh nylon cloth and centrifuged at 2000 rpm for 5 min. The decoctate was freeze-dried to give the aqueous decoction extract (13.43%) used for the various tests.

2.4. Phytochemical Investigation

2.4.1. High-Performance Thin-Layer Chromatography

High-performance thin-layer chromatography (HPTLC) was used to detect fla-

vonoids and tannins in the extracts. It was carried out on chromatoplates (60 F_{254}, 10 × 5 cm, glass support 10 × 20 cm, Merck) following the literature [12]. Approximately 20 µL of each extract was streaked with a semi-automatic sample dispenser (CAMAG, Linomat 5, Switzerland) along the baseline 8 mm 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, (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.

2.4.2. Determination of Total Phenolic Compounds

The total phenolic content of aqueous extract was determined using the Folin-Ciocalteu Reagent (FCR) [5]. Gallic acid was used as the reference compound to produce the standard curve. Briefly, 25 µL of sample at a concentration of 1 mg/mL was mixed with 125 µL of FCR. 100 µL of 7.5% w/v sodium carbonate solution was added to the mixture. After 1 h, 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.

2.4.3. Determination of Flavonoid Compound

Total flavonoid content was assessed using an aluminum chloride reagent [5]. A standard calibration curve was plotted with quercetin as the reference. Briefly, 100 µL of each extract (1 mg/mL) was mixed with 100 µL of a 2% w/v Aluminium trichloride solution. After 10 min, absorbance was measured using a mass spectrometer. After 10 min, absorbance at 415 nm was measured using a spectrophotometer (Epoch Biotek, USA). Results were expressed as mg quercetin equivalent (QE)/g dry extract.

2.5. Assessment of Antioxidant Properties

2.5.1. DPPH• Essay

Free radical scavenging activity by aqueous extract of *D. mespiliformis* leaves and Trolox was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as previously described [13]. Briefly, 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:

$$\text{Inhibition (\%)} = [(Ac - Ae)/Ac] \times 100.$$

Ae and Ac represent the absorbances of the extract/Trolox 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

2.5.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed on the aqueous extract of *D. mespiliformis* leaves 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. Distilled water (0.625 mL) and $FeCl_3$ solution (0.125 mL, 0.1%) were added to the supernatant (0.625 mL), and absorbance was measured at 700 nm using a spectrophotometer (Agilent, Santa Clara, CA) equipped with ChemStation UV-visible software. Ascorbic acid was used to plot the calibration curve. The FRAP activity of the samples was expressed as mmol Ascorbic acid equivalent/gram dry extract.

2.5.3. ABTS• + Assay

The ABTS free radical scavenging activity of the aqueous extract of *D. mespiliformis* leaves and Trolox was assessed using the procedure described previously [5]. In a volume of 5 mL of distilled water, 19.2 mg of ABTS were dissolved. A mass of 3.312 mg potassium persulphate was added to the ABTS solution at 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 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 the extract or reference was added to each well. After incubation for 30 min at 25˚C, absorbances were read against a blank at 415 nm using an Agilent 8453 spectrophotometer. Measurements were performed in triplicate. The percentage inhibition of absorbance at 415 nm was calculated according to the formula:

$$\text{\% Inhibition} = [(A0 - A)/A0] \times 100.$$

A0 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

2.5.4. Lipid Peroxidation Inhibition (LPO) Assay

The lipid peroxidation activity of rat liver was determined using 2-thiobarbituric

acid [5]. $FeCl_2$-H_2O_2 was used to induce peroxidation of liver homogenate. A 0.2 mL volume of extract at a concentration of 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 at 37˚C for 60 min, 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 − (A1 − A2)/A0] × 100.

A1 is the absorbance of the control (without sample); A2 is the absorbance with the sample; A0 is the absorbance without liver homogenate.

2.6. Anti-Inflammatory Activity

2.6.1. 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 [5]. A 96-well microplate was used to perform the sPLA2 inhibition assay. A final concentration of 100 µg/mL of aqueous extract 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] × 100

AE: Absorbance of enzyme assay—Absorbance of blank; AI: Absorbance of inhibition assay—Absorbance of blank.

2.6.2. Lipoxygenase Inhibition Assay

Lipoxygenase inhibition was determined using linoleic acid (1.25 mM) as substrate [5]. Inhibitors (extract/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:

$$\text{Inhibition (\%)} = [(AE - AI)/AE] \times 100.$$

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

2.7. Experimental Animals

Female NMRI (Naval Medicinal Research Institute) mice and Wistar rats (both sexes) with average weights of 27 ± 4 g and 240 ± 15 g, respectively from the animal house of the "Institut de Recherche en Sciences de la Santé/Centre Nationalde laRecherche Scientifique et Technologique (IRSS/CNRST), Burkina Fa-

so" were used. The animals were placed in an enclosure at a temperature of 21˚C - 23˚C with a relative humidity of 50% - 60% and subjected to the light/dark cycle of 12 h/12h according to the rearing conditions of these species. Water and standard laboratory pellets enriched with proteins (29%) 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 [5] [14].

2.8. Acute Oral Toxicity

The acute oral toxicity test for aqueous extract of *Diospyros mespiliformis* leaves was performed on female NMRI (Naval Medicinal Research Institute) mice in accordance with OECD guideline 423 [15] [16]. Two (02) batches, each consisting of three mice, were made up and placed separately in polypropylene cages: a control batch of three mice and a test batch of three mice. After fasting for 3 h, the extract was administered by gavage using an esophageal tube in a single dose to the test mice. A dose of 2000 mg/kg body weight (bw) of the extract was chosen as the starting dose. The white 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 toxidrome (signs of toxicity) such as changes in skin and fur, eyes, mucous membranes, excitement, hyperventilation, vomiting, convulsions, salivation, diarrhea, sleep, and coma. The weight of each mouse and the quantities of water and food consumed were measured every 2 days during the 2 weeks 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 (test batch and control batch).

2.9. Antispasmodic Effect of Aqueous Extracts on Isolated Rat Duodenum

The protocol used has been described by Boly *et al.* [17]. 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), Glucose (1 g) and $CaCl_2$ (0.2 g) in 1L of distilled water]. A 1.5 cm 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 intestine. The physiological 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 aqueous extract or vehicle (distilled water) are administered 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.

2.10. Statistical Analysis

Values are given as arithmetic means ± SEM and means ± SD. Graphics and significance of differences between means were conducted by GraphPad Prism in version 8.4.3 Software, San Diego, CA. Student's T-test and two-way ANOVA followed by Bonferroni multiple comparison tests for comparisons were used.

3. Results

3.1. Phytochemical Investigation

3.1.1. Compounds Revealed by HPTLC

The phytochemical analysis of aqueous extract of *D. mespiliformis* leaves highlighted the presence of saponosides, steroids and triterpenes, flavonoids, and tannins (**Figure 1**).

3.1.2. Total Phenolic and Flavonoid Contents in *D. mespiliformis* Leaves Extract

The total phenolic and flavonoid contents in aqueous extract of *D. mespiliformis* leaves are been respectively 70.59 ± 3.20 mg TAE/g and 31.57 ± 0.78 mg QE/g. 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.

3.2. Antioxidant Activity

The antioxidant activity of aqueous extract of *Diospyros mespiliformis* leaves is shown in **Table 1**. The percentage inhibition of the ABTS test was 7.53 ± 0.08 µg/mL with a better activity for Trolox (3.78 ± 0.21 µg/mL). As for the DPPH radical reduction, the inhibition percentages were 29.47 ± 0.06 µg/mL. Significance was obtained between the percentage inhibition of extract and Trolox whose percentage inhibition was 6.34 ± 0.04 ($p < 0.05$). The ferric ion reduction capacity (FRAP) of the extract varied from 1128.83 ± 4.82 mol EAA/g. The lipid

peroxidation inhibitory (LPO) power of the extract was expressed as a percentage (%) (at 100 µg/mL) with 32.30% ± 1.60% for aqueous extract, and 48.11% ± 3.88% for the Trolox. The value of the aqueous decoction extract was lower than Trolox.

3.3. Anti-Inflammatory Activity *in Vitro*

The evaluation of the *in vitro* anti-inflammatory activity of the extract by inhibiting 15-lipoxygenase and Phospholipase A_2 is recorded in Table 2. In terms of inhibition on 15-lipoxygenase, the Zileuton used as a reference compound in this inhibition test presented a better IC_{50} (2.92 ± 0.32 µg/mL) than the extract, 15.27 ± 1.41 µg/mL. Phospholipase A_2 activity expressed as a percentage of inhibition showed that the aqueous decoction had a low, statistically significant inhibition compared to Betamethasone.

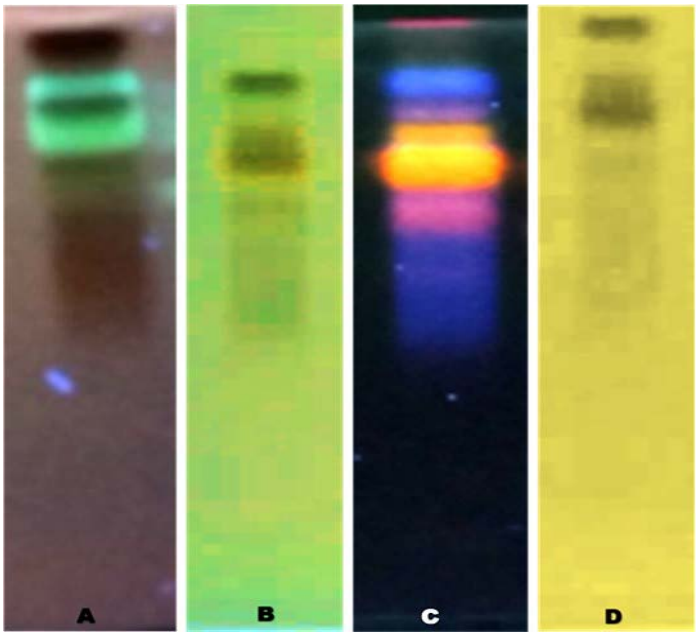

Figure 1. Phytochemical profile of aqueous extract of *D. mespiliformis* leaves revealed by HPTLC. (A) Saponosides; (B) Sterols-triterpenes; (C) Flavonoids; (D) Tannins.

Table 1. *In vitro* antioxidant activity of aqueous extract of *Diospyros mespiliformis* leaves.

Extraits	ABTS		DPPH		FRAP	LPO
	IC_{50} (µg/mL)	ARP	IC $_{50}$ (µg/mL)	ARP	mol EAA/g	Inhibition (%) (at 100 µg/mL)
Aqueous decoction	7.53 ± 0.08***	0.13	29.47 ± 0.06***	0.034	1128.83 ± 4.82	32.30 ± 1.60**
Trolox	3.78 ± 0.21	0.26	6.34 ± 0.04	0.16	-----	48.11 ± 3.88

IC_{50}: Inhibition concentration 50%; ARP: Anti-free radical power; n = 3; **p < 0.01; ***p < 0.001 vs Trolox; EAA: Ascorbic acid equivalent.

Table 2. 15-lipoxygenase and phospholipase A_2 inhibitory activity of aqueous extract of *Diospyros mespiliformis* leaves.

Extracts	15-lipoxygenase IC_{50} (µg/mL)	Phospholipase A_2 inhibition (%)
Aqueous decoction	15.27 ± 1.41***	19.49 ± 2.36###
Zileuton	2.92 ± 0.32	----
Betamethasone	----	35.39 ± 3.31

n = 3; ***p < 0.001 vs zileuton; ###p < 0.001 vs betamethasone.

3.4. Biological Activities

3.4.1. Acute Oral Toxicity

The dose of 2000 mg/kg body weight (bw) showed no mortality or remarkable behavioral changes in female mice at the first and second stages of administration of the aqueous extracts (Table 3).

1) Changes in Body Weight, Food, and Water Consumption of Mice

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), and a single dose (2000 mg/kg) of the aqueous decoctate. There was no statistically significant difference in body weight gain between the treated and control batches. The same was true for feed and water consumption ($p > 0.05$).

2) 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 aqueous decocted of *D. mespiliformis* leaves (2000 mg/kg) showed that there were no lesions, nor any change in color or appearance of the various organs. Figure 3 shows the relative organ weights of batches of control mice and mice treated with the extract. No statistically significant variation was observed between the relative organ weights of control and treated batches.

3.4.2. Antispasmodic Effect of Extract on Isolated Rat Duodenum

The results of the relaxation of the isolated rat duodenum using the extract and vehicle are shown in Figure 4. Figure 4A shows the relaxation curves of the extract and vehicle (control) on the duodenum precontracted with $BaCl_2$, while the histogram (Figure 4B) gives the maximum effects (Emax = 72.78% ± 12.11% and Emax = 5.88% ± 4.40%). A statistically significant difference was noted between these two effects. Similar relaxation results from aqueous extract and vehicle (control) on isolated ACh-precontracted rat duodenum are shown in Figure 5A. With a statistically significant difference, the maximum effects of extract and vehicle were 79.18% ± 9.33% and 10.91% ± 7.47%, respectively (Figure 5B).

4. Discussion

Traditional medicine, or natural remedies using different parts of plants, has been used since ancient times to treat a variety of illnesses. This practice has

been identified as a cost-effective method that can be applied to the treatment of gastrointestinal disorders [18]. The leaves of *Diospyros mespiliformis* are widely

Table 3. Mortality of female mice administered a single dose (2000 mg/kg) of the extract (n = 6).

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

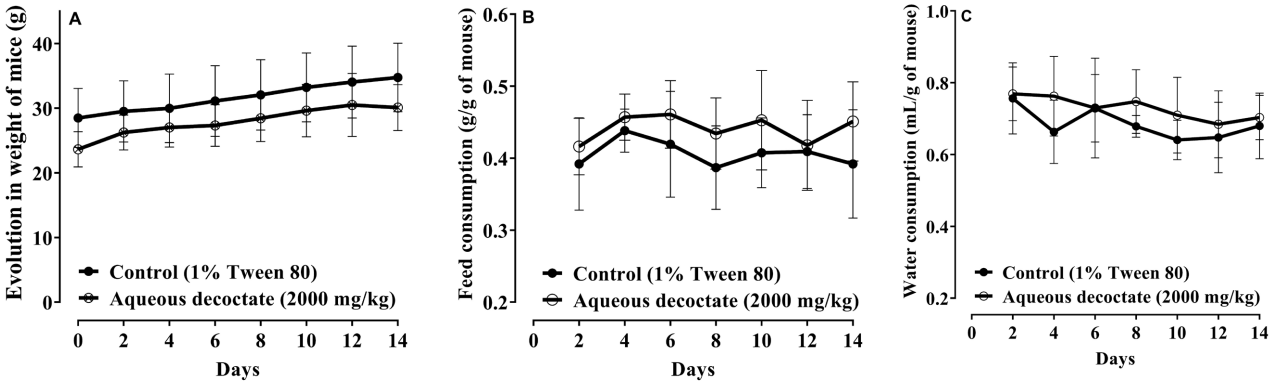

Figure 2. Change in weight (A) and feed (B) and water (C) consumption of female mice from extract test and control batches during 14 days of follow-up; n = 6.

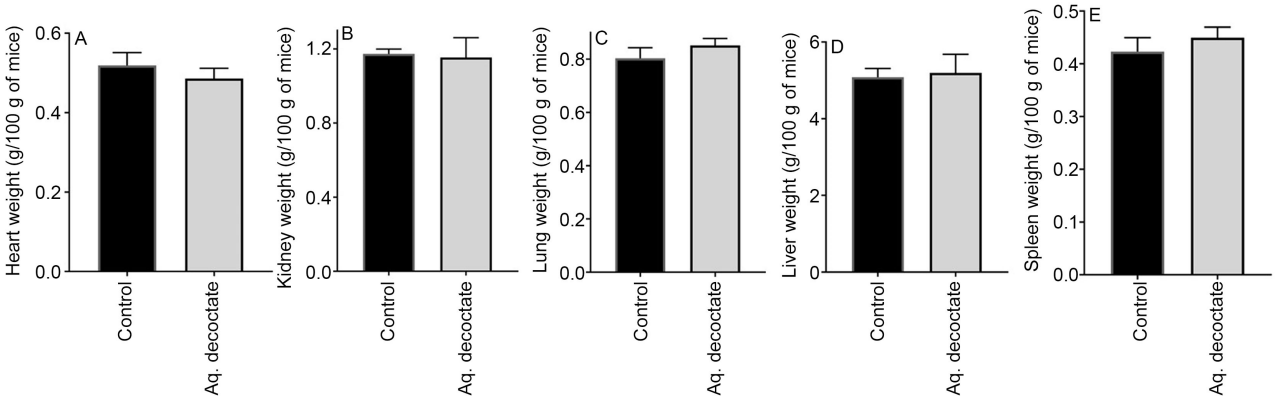

Figure 3. Relative organ weights of control mice and mice treated with a single dose of the aqueous decoctate of *D. mespiliformis* leaves.

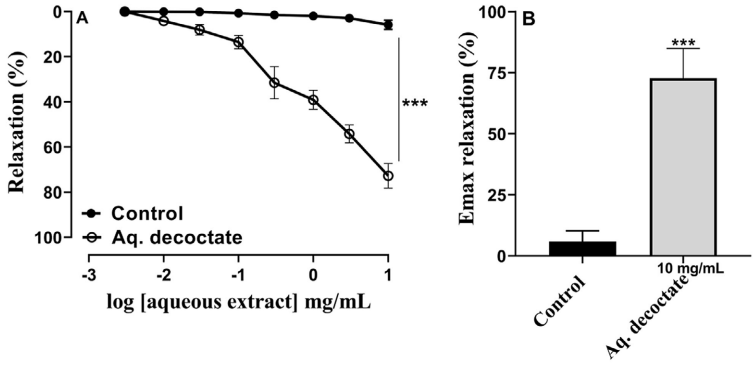

Figure 4. Relaxation curves for aqueous extract of *D. mespiliformis* leaves on isolated $BaCl_2$-precontracted rat duodenum (A) and histogram of maximum relaxation effect (B); n = 5 ; ***p < 0.001 vs control.

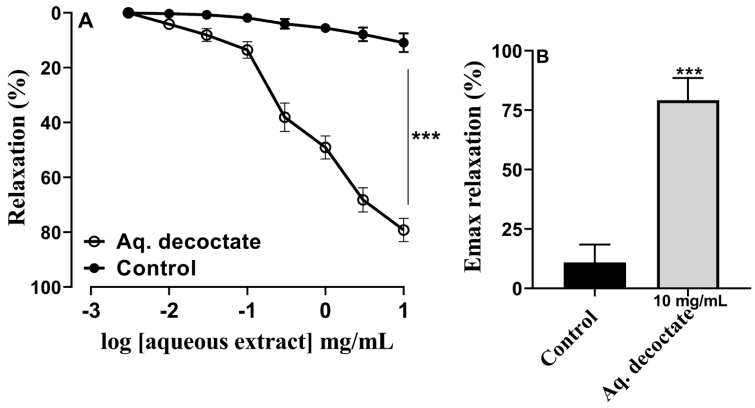

Figure 5. Relaxation curves for aqueous extract of *D. mespiliformis* leaves on isolated ACh-precontracted rat duodenum (A) and histogram of maximum relaxation effect (D), n = 5; ***p < 0.001 vs control.

used as an antispasmodic [4]. The present study aimed to provide scientific evidence for the widespread use of the plant in the treatment of diarrhoea in alternative medicine. Indeed, the phytochemical investigation of *Diospyros mespiliformis* leaves extract is comparable to that reported in studies showing that *D. mespiliformis* leaves contain flavonoids, saponosides, sterols and terpenes as well as tannins [4] [19]. These compounds stabilise reactive oxygen species and eliminate superoxides directly, while other flavonoids can trap the highly reactive oxygen radical known as peroxynitrite [5]. Secondary metabolites have therapeutic properties such as antidiarrhoeal or antispasmodic. This is the case with flavonoids, which have anti-inflammatory and anti-spasmodic properties. Tannins are powerful antioxidants (proton donors to lipid free radicals) with anti-inflammatory, anti-diarrhoeal, anti-parasitic, antibacterial, antifungal, and antiviral activities [20]. In addition, the extracts contained total phenolic contents of around 70.59 ± 3.20 mg TAE/g for the Aqueous decoction, 84.15 ± 1.73 mg TAE/g for the Ethyl acetate fraction and 89.67 ± 2.35 mg TAE/g for the Residual aqueous fraction. Previous studies have shown higher levels of total phenolics in the aqueous, methanolic, ethanolic and petroleum ether extracts of *Diospyros*

mespiliformis leaves [4] [21]. These compounds are known for their antispasmodic properties [22]. The properties of phenolic compounds, in particular flavonoids, tannins and triterpenes, are mediated by the scavenging of free radicals, the inhibition of certain pro-inflammatory enzymes, the chelation of heavy metals involved in the production of free radicals and the regulation or protection of the antioxidant defense system [23] [24]. In addition, the anti-free radical activity and the reduction in the formation of lipid peroxides show that *D. mespiliformis* leaves extract has antioxidant properties. In fact, the antioxidant properties of *D. mespiliformis* leaves extracts have already been demonstrated [4] [25] [26].

Furthermore, in the spasmodic mechanism, inflammation of the viscera cannot be ignored, hence the search for anti-inflammatory properties in extracts for this medication. Overall, the aqueous extract showed good inhibition of 15-Lipoxygenase and Phospholipase A2. These anti-inflammatory activities of the aqueous decoctate were not as good as those of Zileuton and Betamethasone. These results confirm the anti-inflammatory properties of plant extracts [26]. The presences of flavonoids and triterpenes in the extracts are known for their ability to inhibit pro-inflammatory enzymes [5] [27]. To ensure the safe use of leaves extract from this plant, we assessed acute oral toxicity. The results showed that acute oral administration of the aqueous extract of *Diospyros mespiliformis* leaves (2000 mg/kg bw) produced no mortality or visible changes in behaviour or any other physiological activity and indicated that the LD_{50} of this extract was greater than 5 g/kg bw in NMRI female mice, according to the United Nations Globally Harmonised System [28]. These results are in agreement with the literature, which had shown that the methanolic extract of the leaves and trunk barks of *Diospyros mespiliformis*, as well as their hexane, ethyl acetate and butanol fractions, can be consumed safely [29].

Pharmacological results showed that *D. mespiliformis* leaves aqueous extract have muscle relaxant properties on isolated rat duodenum after stimulation of muscarinic receptors by ACh with 79.18% ± 9.33% of efficacy. ACh induces a significant positive tonotropic effect marked by a contracture with plateau contractile activity reflecting the increased peristalsis of the gastrointestinal tract. The contraction induced by ACh is linked to the activation of M_3 muscarinic receptors on the smooth muscle coupled to G proteins, leading via Inositol Triphosphate (IP_3) to the release of intracellular Ca^{2+} [30] [31]. This curative experiment shows that aqueous extract of *D. mespiliformis* leaves have anticholinergic properties. This effect can be explained by the presence in the extract of certain chemical groups such as tannins, flavonoids, saponosides and terpenoids, which directly or indirectly oppose the actions of acetylcholine. These compounds are known for their anti-diarrhoeal properties [22] [32]. Furthermore, these results are in line with the literature, which has documented the anti-diarrhoeal effects of a decoction of *D. mespiliformis* leaves, traditionally used in Ghana and Nigeria [4].

In smooth muscle cells, the cytoplasmic increase in calcium concentration is

the primary stimulus for contraction, which is generally the result of both intracellular release of stored calcium and the influx of extracellular calcium [33]. The concentration-dependent antispasmodic effect of the aqueous decoctate of *D. mespiliformis* leaves on the contractile activity of the isolated intestine could be the result of calcium capture by phosphorylated proteins under the influence of a protein kinase activated by cAMP. It may also be due to an inhibition of calcium influx or an increase in calcium efflux without any change in influx, causing vasodilatation and relaxation of gastrointestinal smooth muscle [22] [34]. In addition, the extract inhibits $BaCl_2$-induced smooth muscle contraction. Indeed, the aqueous extract at concentrations of 0.03 - 10 mg/mL induced a relaxing effect on the rat duodenum by significatively and concentration-dependently reducing contractions. The extract could therefore have a musculotropic effect like papaverine, by inhibiting the function of phosphodiesterase [17]. All these different mechanisms of action could explain the muscle relaxant effect of the aqueous extract. However, the mechanism of action of this extract has not been studied in depth. The same applies to the evaluation of its anti-diarrhoeal properties *in vivo*. Future work should therefore focus on the mechanics of the extract, its bioguided fractionation and an assessment of its medium- and long-term oral toxicity.

5. Conclusion

We are not aware of any work on the antispasmodic properties of the aqueous decoction of *D. mespiliformis* leaves. Results have shown that extract of *D. mespiliformis* leaves inhibits the contractile actions of intestinal muscle by ACh and $BaCl_2$. These effects are thought to be mediated by phytoconstituents such as tannins, flavonoids, saponosides, sterols and terpenoids, which also have antioxidant and anti-inflammatory effects. The extract is non-toxic in a high single dose. Thus, the present study contributes to new knowledge of the antispasmodic 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.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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