Original Article

Dose-dependent Action of *Zingiber officinale* on Colonic Dysmotility and Ex Vivo Spontaneous Intestinal Contraction Modulation

Dose-Response:
An International Journal
July-September 2022:1–17
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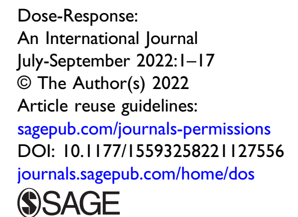

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Abstract

Ginger (*Zingiber officinale*) rhizomes are commonly used in foods and employed for many ailments including gastrointestinal disorders. Our main objective was to evaluate the effect of *Zingiber officinale* aqueous extract (ZOAE) on gastrointestinal (GI) physiological motility and colonic dysmotility. Thereby, *Wistar* rats were given loperamide (LP, 3 mg/kg, b.w.) and ZOAE (75, 150, and 300 mg/kg, b.w.) or yohimbine (YOH, 2 mg/kg, b.w.). ZOAE-action on intestinal secretion was assessed using Ussing chamber technique and intestinal motility with isometric transducer. GI-transit (GIT) and gastric emptying (GE) were evaluated with the charcoal meal test and the red phenol methods. ZOAE-bioactive components were analyzed by liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS). Constipation was induced with LP and the different indicators such as stool composition, GIT, oxidative stress biological parameters, and colonic mucosa histological alteration were performed. Anti-constipation effect of ZOAE was confirmed on stool composition, GIT (53.42% to 85.57%), GE (55.47% to 98.88%), and re-established oxidative balance. ZOAE induces an amplitude increase of spontaneous intestinal contraction with EC50 of 10.52 μg/mL. No effect of ZOAE was observed on electrogenic transport of intestinal fluid. These findings suggest that ZOAE-bioactive candidates might exert an anti-constipation action and spontaneous intestinal contraction modulation.

Keywords

ginger rhizomes, colonic dysmotility, spontaneous intestinal contraction, rats, *Zingiber officinale*, gastro-intestinal transit, antioxidant activity, intestinal contraction

Introduction

Constipation signs include slow bowel movement, extremely dehydrated feces, difficulty of fecal elimination, abundant demanded bowel motilities, visceral bloating due to gas accumulation, and abdominal displeasure. In both children and adults, the functional constipation pathophysiology is designed to be multifactorial. It might arise from various factors, including nutritional status, synthetic components such as the use of opioids, and emotional strain and pressure.[1] Recent researches have showed that functional constipation was related with other diverse factors, such as the change of gastrointestinal nerve cells, neuro-muscular diseases, neurotransmitter, and dysbiosis.[2]

The imbalance between free radicals and antioxidants or the oxidative damage was shown in many studies to play a

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Received 18 June 2022; accepted 3 September 2022

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crucial role in the development of various functional gastrointestinal disorders (FGDs) such as constipation. Indeed, continual constipation child produces oxidative injury and potential free radical destruction. These impairments were associated with enzymatic/non-enzymatic antioxidants depletion as well as the overproduction of the major toxic by-products of lipid peroxidation and reactive oxygen species (ROS) accumulation like hydrogen peroxide (H_2O_2).[3]

Laxative chemical drugs with serious side effects are recommended to trigger intestinal movements and help patients pass feces. For this, the perfect manner for the management of this bowel slow pathogenesis is producing some habitual changes to assimilate more natural laxatives as fiber foods to relieve constipation, drinking enough liquids, especially water, and adding regularly exercising.[4]

Added to that, recently, several natural products exerting laxative actions have honored highest consideration as new therapeutic approaches for constipation management and its combined disruptions, despite various researches required to investigate the potential mechanisms of pharmacological targets.[5] In this context, many studies have revealed the ability of bioactive compounds of natural products especially with great antioxidant power may relieve chronic constipation and its related symptoms. These bio-compounds are gaining a potent concern in the biopharmaceutical sector and encouraging the seeking for new important roots of bioactive components. In fact, several medicinal plants containing alkaloids, tannins, coumarins such as *Aloe ferox*,[6] *Malva sylvestris*,[7] *Urginea indica Kunth*,[8] *Ficus carica*,[9] and others certainly declined affections related to LP-caused slow colonic motility as the three stool parameters, gastric-emptying time, and small intestinal transit time as well as oxidative stress parameters management in animal models.

Zingiber officinale is a plant species native to India belonging to the *Zingiberaceae* family.[10] The rhizomes of which are used worldwide not only as a spice but also in traditional medicine.[10,11] Rhizomes have been used frequently for a very long time in traditional medicine to relieve muscle pain, diabetes, nervous diseases, stroke, hypertension, dementia, migraine, and asthma and obviously used to treat several gastrointestinal disorders such as constipation, diarrhea, nausea, vomiting, dyspepsia, gastric ulcerations, bloating, belching, gastritis, epigastric discomfort, and indigestion.[10,12,13] Its phytochemical bioactive compounds were identified to possess strong anti-oxidant[14,15] and anti-inflammatory activities.[14,16]

Currently, the requirement and use of ginger and its various by-products such as gingerbread, ginger cake, ginger coffee, ginger drink, ginger oil, ginger spice, and ginger syrup as well as pharmaceutical, food, and other associated industrial productions have strongly raised.[17]

However, it is not clear whether the *Zingiber officinale* aqueous extract (ZOAE) can alleviate constipation. Therefore, we used LP-induced constipation in rats to evaluate its effectively relieving effect as well as the possible associated mechanism of actions through the study of its in vivo and in vitro antioxidant activities as well as its effect on ex vivo spontaneous intestinal contraction.

Materials and Methods

Drugs and Chemicals

Gum arabic, charcoal meal, red phenol, methyl cellulose, sodium hydroxide (NaOH), NaCl, hydrochloric acid (HCl), 2,2-diphényl 1-picrylhydrazyl (DPPH), butylhydroxyanisol (BHA), 2-thiobarbituric (TBA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), methanol, ether, epinephrine, bovine catalase, Folin Ciocalteu, GSH, tris, hydrogen peroxide (H_2O_2), and yohimbine were obtained from sigma chemicals Co (Sigma-AldrichGmbH, Steinheim, Germany). Loperamide hydrochloride was purchased from local pharmacy and the other chemicals were used of analytical grade.

Fresh Plant Material and Extract Preparation

The rhizomes of fresh plant (*Zingiber officinale*) of Chinese origin were purchased from the regional market in Tunisia. After washing, they were cut into rings and desiccated at 40°C with air circulation for 72 hours. The dried rings were ground using a laboratory blender until a fine powder was obtained. 1 g of this material was shaken for 24 hours in 20 mL of distilled water. The obtained aqueous extract was filtered using Whatman No 1 (70 mm) filter paper[18] (extraction yield = 27%) and stored at −80°C until use.

Zingiber officinale *Aqueous Extract-Phenolic Compounds Identification by Liquid Chromatography-High Resolution Electrospray Ionization Mass Spectrometry (LC-HRESIMS) Analysis*

100 mL of 10% methanol was used to dissolve 100 mg of ZOAE. Then the mixture was filtered and 1 mL was transferred into LC-MS vials for analysis. An opposite phase column (Pursuit XRs ULTRA 2.8, C18, 100*2 mm, Agilent Technologies, United Kingdom) was used to conduct HPLC surveys. 20 mL of prepared sample were injected at a column temperature set at 30°C. The mobile phases consisted of .1% formic acid in water (A) and .1% formic acid in methanol (B). A gradient program was used for isolation at a flow rate of 1 $mL.min^{-1}$. The mobile phases consisted of an initial composition at 100% solvent A, with a gradient of 100% solvent B for 20 minutes, maintained at 100% solvent B for 5 minutes and at 100% solvent A for 25 minutes.

The drying gas flow rate was 1 $mL.min^{-1}$ at 320°C. MS was used in positive ion mode in a mass range from 100 to 2000 m/z. High resolution mass spectral data was obtained on a Thermo Instruments ESI-MS system (LTQ XL/LTQ Orbitrap Discovery, UK) connected to a Thermo Instruments

HPLC system (Accela PDA detector, Accela PDA auto-sampler and pump Accela).[19]

Phytochemical Compounds Contents and ZOAE-Antioxidant Activity

Total Phenol Quantity Analysis. The determination of total polyphenols was carried out according to the method of Dewanto et al.[20] Briefly, a volume of .5 mL of the diluted extract was mixed with .5 mL of Folin Ciocalteu reagent (1/10) and then neutralized with 1 mL of 20% sodium carbonate (w/v). After 1 hour incubation at room temperature, the absorbance was measured at 765 nm against a blank using ultraviolet–visible spectrophotometer. The concentration of total polyphenols contained in the extract is calculated by referring to a calibration curve obtained using gallic acid as standard. The results obtained are expressed in milligram of gallic acid equivalent per gram of dry matter (mg GAE/g of DM).

Total Flavonoid Amount Exploration. The estimation of total flavonoids was carried out using the aluminum trichloride method. The protocol used is described by Zhishen et al[21] and Kim et al[22] with a few small modifications. An aliquot of 400 µl of the appropriately diluted extract was added to 120 µL of $NaNO_2$ (5%). After 5 minutes, 120 µL of $AlCl_3$ (10%) have been added, and the medium is mixed thoroughly. After 6 minutes, 800 µL of NaOH (1M) was added to the medium. The absorbance is read immediately at 510 nm against a blank using ultraviolet–visible spectrophotometer. The concentration of total flavonoids contained in the extract is calculated by referring to a calibration curve obtained using quercetin as standard. The content of flavonoids is expressed in milligram of quercetin equivalent per gram of dry matter (mg QE/g of DM).

Condensed Tannins Level Determination. The content of condensed tannins was determined according to the vanillin methods described by Julkunen-Titto.[23] A volume of 50 µL of the appropriately diluted extract was added to 1500 µL of the vanillin/methanol solution (4%) and then thoroughly mixed. Then 750 µL of concentrated hydrochloric acid (36%) was added.

The mixture obtained was incubated at room temperature for 20 minutes. The absorbance is measured at 550 nm against a blank. The concentration of condensed tannins in the extract is calculated by referring to a calibration curve obtained using catechin as standard. The results obtained are expressed in milligram of catechin equivalent per gram of dry matter (mg CE/g of DM).

Antioxidant Activity of ZOAE. The antioxidant activity of ZOAE was achieved through 2 mechanisms: the ability to scavenge a free radical (DPPH) and chelating power on ferrous ions:

2,2-diphényl 1-picrylhydrazyl Free Radical Scavenging Ability of ZOAE. The anti-radical capacity evaluation of the extract was carried out using the method of Bersuder et al.[24] Diverse concentrations of 500 µL of ZOAE were brought into contact with 375 µL of 100% ethanol and 125 µL of .02 mM DPPH in 100% ethanol. Following vigorous shaking, the mixture is incubated for 60 minutes in darkness and at ambient temperature then the reduction of $DPPH^{\bullet}$ radical was checked at 517 nm. The control sample was performed in like manner, using distilled water instead of the ZOAE.

The free radical of DPPH is estimated as a percentage of inhibition according to the following expression: $I (\%) = [(Ab + Ac - As)/Ac)] \times 100$.

Ab and Ac represent the blank absorbance and the control reaction tubes, respectively, and As is the absorbance of the sample. The extract concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages compared with concentrations of the extract.

Ferrous Ion-Chelating Ability Assay

The Fe^{2+} chelation capacity of ZOAE was determined using the method of Decker and Welch[25] with a slight modification. Briefly, 50 µL of $FeCl_2$-$4H_2O$ solution (2 mM) was added to 100 µL of ZOAE (.5-5 mg/mL) diluted in 450 µL of distilled water. The reaction was started by the inclusion of 5 mM ferrozine (.2 mL) and the combined solution was shaken eagerly and then incubated for 10 minutes at room temperature. The absorbance was examined at 562 nm and the Fe^{2+} chelation capacity was then estimated corresponding to the control sampled. The percentage of Fe^{2+} chelation capacity (%) using the following formula:

$$\%\,\text{Chelation} = [(\text{Ac} + \text{Ab} - \text{As})/\text{Ac}] \times 100$$

Ac represents the absorbance of the control reaction. Ab is the absorbance of the blank and As is the absorbance of the sample, respectively.

Used Animals for Experiments

Adult male rats of the Wistar strain (Ten weeks old, weighting 180–220 g) and male mice (weighting 20-30 g) were purchased from the Society of Pharmaceutical Industries of Tunisia (SIPHAT, Ben-Arous, Tunisia). The animals were housed six per cage with *ad libitum* access to water and a standard food (Badr-Utique-TN). They are maintained under standard conditions of temperature 22 ± 2°C, relative humidity of 50%, and 12 light/dark cycles in the animal house of the Higher Institute of Biotechnology of Beja and used for toxicity and constipation studies.

Male mice of the C57BL/6JRj strain (7 weeks old, weighing 20-25 g) from Janviers SASA (Route des chênes, Le Genest-st-Isle, St Berthevin, France) were grouped in polycarbonate cages and acclimatized for 1 week under the

following conditions (22–26°C, ventilation, and 12/12 light/dark cycle) with free access to water and food in the animal house of the Faculty of Pharmacy, University of Lille, France, and used for intestinal contraction studies. Circadian rhythm can influence important functions in the body, for this, all experiments were performed at the same time (9 h) every day. Moreover, all animal treatments were approved by the Institutional Animal Care and Use Committee of National Institute of Health and performed according with the NIH Guidelines for Care and Use of Laboratory Animals.[26]

Acute Toxicity Study

The acute toxicity was evaluated, by administering orally, increasing doses of the ZOAE ranging from 10 to 3200 mg/kg, b.w. to mice divided into 8 groups of 10 animals each. A control group was treated with 10 mL/kg of NaCl 0.9%. The animals were examined every 30 minutes for the first 6 hours on the first day, then once per day for 48 hours. These observations were based on mortality, mobility, respiratory changes, and poor appetite.

GI-Transit Measurement

GI-motility was evaluated using the charcoal meal method.[27] The rats were fasted for 16 hours and divided into 6 groups of 6 animals each: Group 1 served as a negative control and received 1 mL of physiological solution (NaCl, .9%), Groups 2 and 3 received YOH (2 mg/kg, b.w.) and LP (3 mg/kg, b.w.), respectively, and Groups 4, 5, and 6 were treated with different doses of the ZOAE (75, 150, and 300 mg/kg, b.w.). A standard charcoal meal (10% charcoal in 5% gum arabic) was administered orally using an intra-gastric tube 2 hours after treatment. 30 minutes later, the animals were sacrificed and the distance traveled by the charcoal meal from the pylorus was measured. GIT was expressed as a percentage and calculated according to the following rule:

$$\mathrm{GIT}(\%) = (\text{Distance traveled by charcoal meal} / \text{Length of small intestine})(\mathrm{cm}) \times 100$$

GE Assessment

To measure the ZOAE-action on GE, the method of the red phenol was used.[28] Animals were randomized into six lots of six animals each and treated 1 hour before test-meal [50 mg phenol red in 100 mL aqueous methyl cellulose (1.5%)] as follows: Group 1 served as a negative control and received 1 mL of physiological solution (NaCl, .9%), Groups 2 and 3 received the standard-drug, YOH (2 mg/kg, b.w.), and LP (5 mg/kg, b.w.) and Groups 4, 5, and 6 were treated with various doses of the ZOAE (75, 150, and 300 mg/kg, b.w.). Sixty minutes of receiving of the phenol red, animals were killed. The gastric contents were combined with 100 mL of NaOH (0.1 N). The suspension was allowed to settle for 1 hour at room temperature, and to 2.5 mL of the supernatant .25 mL of trichloroacetic acid 20% (w/v) was added and centrifuged at 1800 g for 20 minutes. The supernatant was finally mixed with 4 mL of NaOH (0.5 N) and the absorbance of the samples was read at 560 nm. The collected phenol red from animal stomachs after the test meal intra-gastric administration was considered as the standard (0% of GE).

The GE rate of percent was determined according to the following formula:

$$\mathrm{GE\ rate}(\%) = (1 - \text{absorbance of treated}/\text{absorbance of standard}) \times 100$$

Obtaining Constipated Rats

The gastric gavage of LP (3 mg/kg, b.w., in .9% sterilized physiological saline) for 1 week was used to induce the slow colonic transit. A total of 36 rats were used for the experiment and they were divided into the following groups:

Group 1: Negative control group (n = 6), in which rats were treated with a physiological solution (10 mL/kg of body weight).
Group 2: Constipated group (n = 6), in which rats were treated with LP (3 mg/kg of body weight).
Group 3: LP + ZOAE 75 (n = 6), in which rats were treated first with LP and 1 hour later treated with 75 mg/kg of body weight of ZOAE.
Group 4: LP + ZOAE 150 (n = 6), in which rats first were treated with LP and 1 hour later treated with 150 mg/kg of body weight of ZOAE.
Group 5: LP + ZOAE 300 (n = 6), in which rats first were treated with LP and 1 hour later treated with 300 mg/kg of body weight of ZOAE.
Group 6: YOH group (n = 6), in which rats were treated first with LP and 1 hour later treated with a standard drug YOH (2 mg/kg of body weight).

The water/food intake was measured during the experimental duration.

The wet/dry weight of the rat fecal pellets was collected on day 5 during 24 hours, and we calculated modifications in these indicators.

The fecal water level was done by putting the fecal samples at 70°C for 24 hours and calculated by the difference between the weight before and after drying.[29]

At the last of the practice, the animals were decapitated and the collected blood was centrifuged at 3000 g/20min/4°C to obtain plasma. The colons of constipated rats were removed and recovered under glace and the mucosae were then homogenized in the Tris-buffered saline (TBS) solution and then centrifuged at 3000 g/15 min/4°C.

The supernatant and the plasma thus obtained were stored at −80°C to be used for biochemical assays.

Ex Vivo Antioxidant Activities of ZOAE

Catalase (CAT) activity was measured according to the Aebi method[30] by following the decrease in the absorbance of the reaction medium (phosphate buffer + plasma or colon mucosa) after addition of H_2O_2 at 240 nm. Superoxide dismutase (SOD) was determined according to the method of Misra and Fridorich.[31] Moreover, the activity of glutathione peroxidase (GPx) was examined based on Flohé and Günzler procedure.[32]

The last components of polyunsaturated fatty acids peroxidation in the cells or the malondialdehyde (MDA) produced following an accumulation of the free radicals during oxidative stress installation. It is measured by the method of Draper et al.[33] Sulfhydryl groups (-SH) level was realized using Ellman's method.[34] Reduced glutathione (GSH) level was accomplished in agreement with the Sedlak and Lindsay method.[35]

Colon Histology

Colonic tissue segments were removed and fixed directly in formaldehyde (10%) after sacrifice for histopathological observations. The samples were subsequently embedded in paraffin and then sectioned into 5 μm thick slices. These sections were stained with hematoxylin and eosin (H&E) solution to study the change in thickness and inflammatory infiltrate of the colonic mucosa, while the secretion of mucus was observed with the staining of the Alcian blue.

Study of Intestinal Contraction and Relaxation

Overnight fasted male mice are subjects to vertebral dislocation. A segment of 5 cm of the jejunum was excised and washed in saline solution under the ice. The forceps were used to strip off precisely the jejunum mesenteric border. The jejunum sections (2 cm) were detached using flushing with a solution of Tyrode whose composition is as follows: NaCl (136.9 mM), KCl (2.7 mM), $CaCl_2$ (1.8 mM), $NaHCO_3$ (11.09 mM), $MgCl_2$ (1.05 mM), NaH_2PO_4 (.42 mM), and glucose (5.5 mM) at pH 7.4. Each tissue was put in a 3 mL organ bath enclosing Tyrode's solution maintained at 37°C ± .5 and providing 95% O_2 and 5% CO_2. A first tension of .5 g was used, and the spontaneous muscular contractility was registered isometrically at the same time utilizing JFD-2 Transducer (Laboratoires TBC, France). Drugs and ZOAE were joined immediately to the organ chamber in volumes not more than 1% of the total bath volume. At the last of the 45-minutes equilibration duration, the actions of various doses of the ZOAE and/or the drugs were evaluated cumulatively with a contact period of 2 minutes for each concentration. The effect on contraction and relaxation of the extract at a concentration of 500 μg/mL against 10^{-6} M carbamylcholine (CarbCh), 25 mM KCl and 10 mM $CaCl_2$ was assessed.

Study of Intestinal ZOAE Secretion in Ussing Chambers

Jejunum tissues have been put in Ussing chambers (exposed area, .30 cm^2) containing on each side 3 mL of a Ringer solution composed of NaCl (115 mM), $NaHCO_3$ (25 mM), $MgCl_2$ (1.2 mM), $CaCl_2$ (1.2 mM), K_2HPO_4 (2.4 mM), and KH_2PO_4 (.4 mM).

Each chamber must be maintained at a constant temperature of 37°C and consecutively gassed by bubbling carbogen (95% O_2–5% CO_2).[36] During mounting, the sample must be well spread out and completely cover the orifice of the Ussing half-chambers. Subsequently, 100 μL of the ZOAE at a concentration of 500 μg/mL was added after forskolin (10 μM) was placed in the serous side. Forskolin causes an increase in the chloride secretion of the cell capacity. It is also used to check the viability and sensitivity of the epithelial tissue. Using electrical parameters, short circuit current (Isc), and conductance (G), it is possible to study the action of ZOAE on secretion through the mouse jejunum.

Statistical Analysis

Pharmacological responses for isolated experiments using n tissues are presented as means ± SEM (standard error of the mean). Graphs of the concentration–response curves were resolved using nonlinear regression and were fitted to the Hill equation by an iterative least-squares method (GraphPad Prism version 8.0 for Windows, GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was realized for the comparison of the diverse actions with the control (Dunnett) and for the multiple-group comparisons (Bonferroni–Dunn). Linear regression was used for food intake and water consumption computed using the trapezoid rule. Statistical significance was set as $P < .05$.

Results

Identification and Quantification of Zingiber Officinale Aqueous Extract Phenolic Compounds With LC-HRESIMS Assay

The characterization of bioactive components in ZOAE was accomplished by LC-HRESIMS and 23 phenolic compounds were experimentally established based on their retention times. The main ones are quinic acid, trans-cinnamic acid, rosmarinic acid, cirsiliol, protocatechuic acid, and p-coumaric acid (Figure 1 and Table 1).

Phenolic Compound Quantities

The result of colorimetric analysis of chemical constituents of ZOAE indicates that the mean polyphenols, flavonoids, and condensed tannins contents were equivalent to 4.14 ± .06 mg GAE/g DM, 2.34 ± .19 mg QE/g DM, and .33 ± .02 mg CE/g DM, respectively (Figure 2).

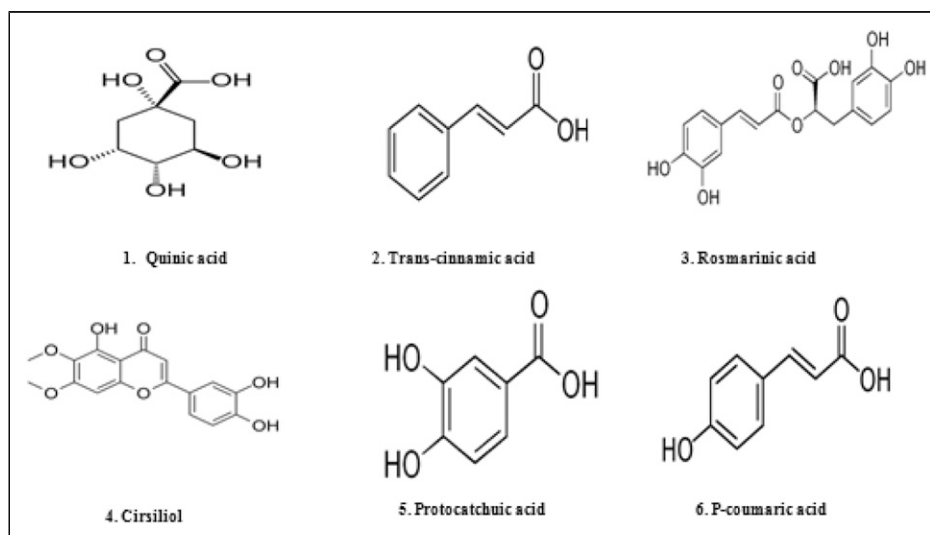

Figure 1. ZOAE-phenolic major compounds.

Table 1. Liquid Chromatography-High Resolution Electrospray Ionization Mass Spectrometry (LC-HRESIMS) Analysis of ZOAE.

	Molecular Formula	PubChem	(M) H m/z	Retention Time	Concentration, ppm
1. Quinic acid	$C_7H_{12}O_6$	6508	191	2.112	147.609
2. Trans-cinnamic acid	$C_9H_8O_2$	444539	147	32.168	75.046
3. Rosmarinic acid	$C_{18}H_{16}O_8$	5281792	359	26.487	47.426
4. Cirsiliol	$C_{17}H_{14}O_7$	160237	329	35.830	10.630
5. Protocatechuic acid	$C_7H_6O_4$	72	153	6.907	8.868
6. P-coumaric acid	$C_9H_8O_3$	637542	163	21.061	7.317
7. Syringic acid	$C_9H_{10}O_5$	10742	197	16.259	3.365
8. Trans-ferulic acid	$C_{10}H_{10}O_4$	445858	193	23.302	3.186
9. Naringin	$C_{27}H_{32}O_{14}$	42428	579	26.258	2.519
10. Caffeic acid	$C_9H_8O_4$	689043	179	14.607	2.442
11. Luteolin-7-o-glucoside	$C_{21}H_{20}O_{11}$	5280637	447	24.859	1.835
12. Apigenin-7-o-glucoside	$C_{21}H_{20}O_{10}$	45933926	431	27.171	.978
13. 4-O-caffeoylquinic acid	$C_{16}H_{18}O_9$	5281780	353	11.634	.798
14. Chlorogenic acid	$C_{16}H_{18}O_9$	1794427	353	11.634	.773
15. Kaempferol	$C_{15}H_{10}O_6$	5280863	285	32.213	.721
16.4,5-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	6474309	515	27.060	.597
17. Acacetin	$C_{16}H_{12}O_5$	5280442	283	40.654	.409
18. Quercetin (quercetin-3-o-rhamnoside)	$C_{21}H_{20}O_{11}$	15939939	447	27.024	.394
19. Hyperoside (quercetin-3-o-galactoside)	$C_{21}H_{20}O_{12}$	15939939	463	24.813	.305
20. Apigenin	$C_{15}H_{10}O_5$	5280443	269	34.762	.277
21. Quercetin	$C_{15}H_{10}O_7$	5280343	301	32.235	.190
22. Luteolin	$C_{15}H_{10}O_6$	5280445	285	35.201	.044
23. Cirsilineol	$C_{18}H_{16}O_7$	162464	343	39.057	.032

2,2-diphényl 1-picrylhydrazyl Scavenging and Metal Ion Chelating Activity of ZOAE

The antiradical power of ZOAE to trap DPPH free radical was evaluated based on the concentration required for 50% inhibition of this radical (IC_{50}). The kinetics of degradation of the DPPH radical as a function of the increasing concentrations of ZOAE and BHA made it possible to specify an IC_{50} of the order of .35 ± .01 mg/mL and .22 ± .01 mg/mL (Table 2).

The chelating capacity extract is measured by monitoring the inhibition of Fe (II)-ferrozine complex formation after incubation of ZOAE, at different concentrations, with divalent iron. The results obtained showed that the inhibition

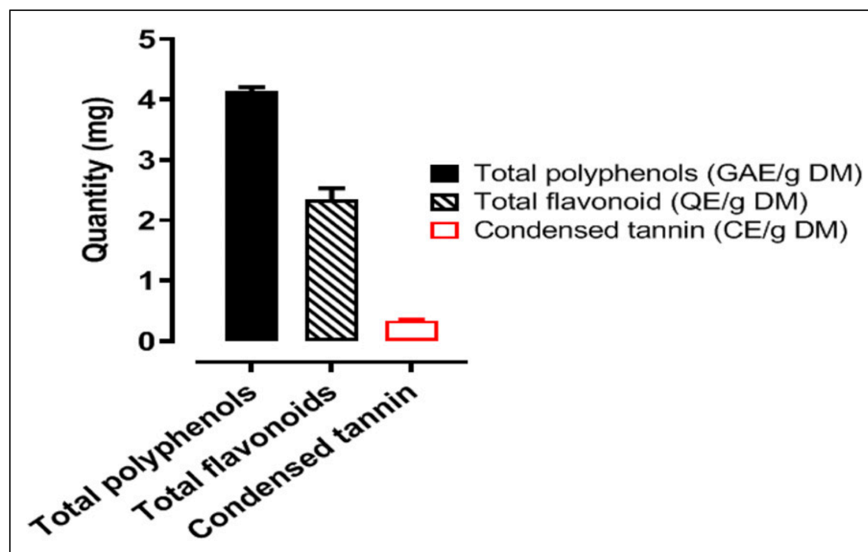

Figure 2. Phytochemical composition of ZOAE.

Table 2. IC_{50} (mg/mL) Values of ZOAE for DPPH and Metal Chelating Tests.

IC_{50}	DPPH Radical	Metal Chelating
EAZO	.35 ± .01	1.99 ± .15
BHA	.22 ± .01	.66 ± .08

DPPH, 2,2-diphényl 1-picrylhydrazyl; BHA, butylhydroxyanisol; ZOAE, *Zingiber officinale* aqueous extract; IC_{50}, the median inhibitory concentration. Data are represented as means ± SD (n = 3).

concentration (IC_{50}) to chelate 50% of Fe^{2+} is equal to 1.99 ± .15 mg/mL and .66 ± .08 mg/mL, respectively, for ZOAE and BHA (Table 2).

Effect of ZOAE on Acute-Toxicity Studies

The acute-toxicity test of ZOAE at the oral limit doses of 10, 50, 100, 200, 400, 800, 1600, and 3200 mg/kg caused no abnormal behavior or mortality in the mice. No signs of toxicity were observed pending the monitoring period. LD_{50} value considered higher than 3200 mg/kg.

Effect of LP and ZOAE on Food Intake and Water Intake

Figure 3 has shown that LP causes a remarkable diminution in these parameters in constipated rats compared to normal rats. However, the treatment with ZOAE considerably increases access to water in a dose-dependent response in comparison to the constipated animals and improves food consumption only at the highest dose (300 mg/kg) compared to the same group. No significant difference ($P > .05$) was detected in the food and water intake between the negative control and the rats treated with ZOAE.

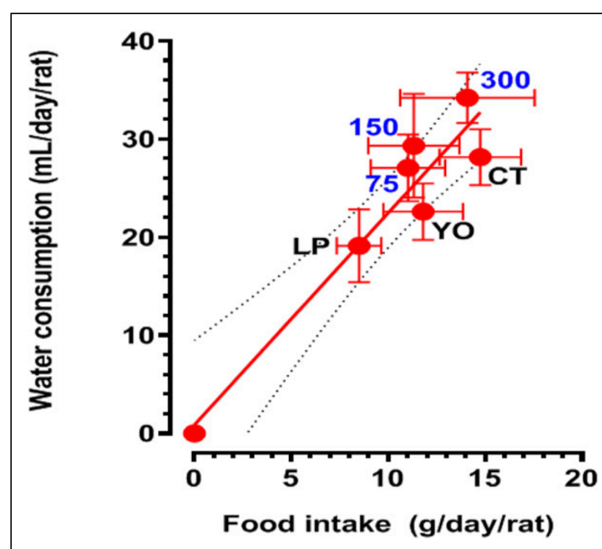

Figure 3. Linear correlation between water consumption and food intake. Animals were treated with NaCl solution (CT, NaCl 0.9%), LP (3 mg/kg, b.w.), YOH (positive control group, 2 mg/kg, b.w.), and different concentrations of ZOAE (75, 150, and 300 mg/kg, b.w.).

Control of Constipation Induced by LP

The effect of LP in inducing constipation is demonstrated in Figure 4. Significant decrease in gastrointestinal movement was observed in LP-group rats compared to the negative control with a reduction of 22.70% (Figure 4A). It also delayed gastric emptying time in constipated rats (%GE= 55.47 ± 1.40%) compared to the negative control (%GE= 70.77 ± 1.23%) (Figure 4A). Oral administration of LP induces a significant reduction in the total number of stools collected on day 5 in constipated rats (n = 38.23 ± 4.88) compared to the negative control group (n = 109.00 ± 5.56) (Figure 4B). A reduction

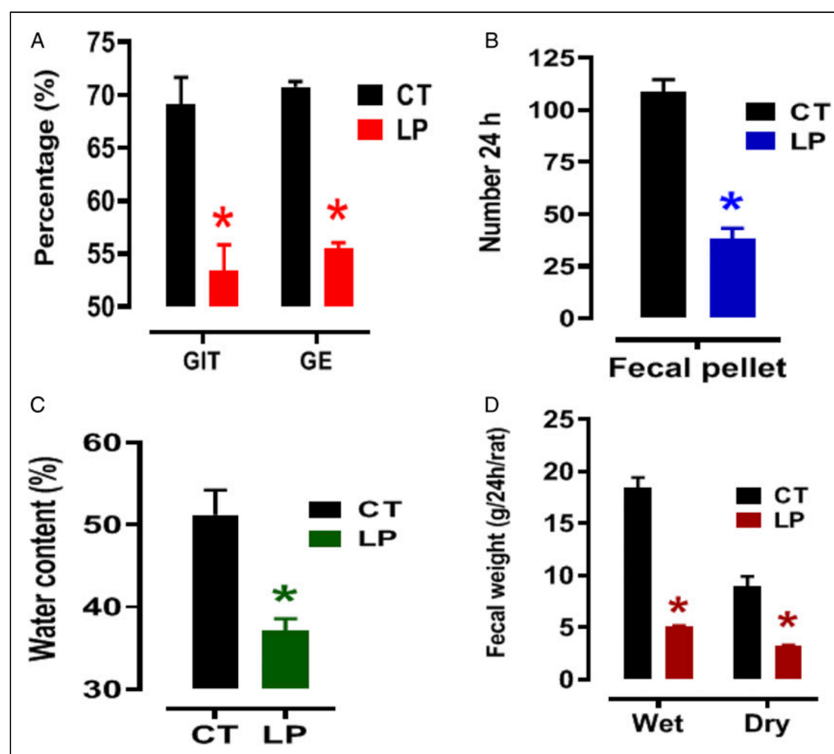

Figure 4. Effect of LP on GIT and GE (Figure 4A), as well as its action on total number of fecal markers [pellet (**B**), water content of stools (**C**), and wet/dry fecal weight (**D**)] discharged over 24 h. Data are expressed as means ± SEM (n = 6). *: $P < .05$ in comparison with the no treated animals (ANOVA test).

of 27.60% in fecal water content was also detected in constipated rats compared to the negative control (Figure 4C). This reduction is proved by a drop in weight of wet and dry stools compared to the control group (Figure 4D).

As shown in Figure 5A, the highest rate of lipid peroxidation was marked in constipated rats at the plasma and colonic level following intoxication by LP. On the other hand, for non-enzymatic antioxidants, LP produced in the colon and plasma a reduction in reduced glutathione (GSH, Figure 5B) and sulfhydryl group (-SH, Figure 5C) activity in LP-treated animals compared to the control group.

For the enzymatic antioxidant, the results presented in Figure 6 showed that loperamide significantly reduced colonic and plasma catalase (Figure 6D), GPx (Figure 6E), and SOD activities (Figure 6F) compared to the control group.

Anti-Constipation Effect of Zingiber officinale *Aqueous Extract*

The treatment with ZOAE accelerated the intestinal transit compared to the constipated rats at all the doses tested (Figure 6A). Likewise for gastric emptying, ZOAE accelerated this process in a dose-dependent manner compared to constipated rats (Figure 6A). The protective effect of ZOAE was demonstrated by increasing the total number of stools while protecting them from dehydration. The latter was reflected by an increase in fecal water content. The highest content was marked at 300 mg/kg, b.w. (Figure 6B). This is confirmed by an increase in the weight of wet stools (Figure 6C).

The increase in MDA caused by LP was significantly attenuated in ZOAE-treated rats with a reduction of 35.82% and 41.91%, respectively, for colon and plasma at the highest used dose (Figure 7A). As shown in Figure 7B, the decrease in plasma and colonic reduced glutathione activity previously observed in constipated rats was significantly improved by ZOAE treatment for all doses and only at the last 2 doses for sulfhydryl group activity in colonic mucosa (Figure 7C).

Catalase activity was improved significantly by treatment with ZOAE in the colonic mucosa at different doses. For plasma, the increase in activity is slight but significant at the highest dose (Figure 7D). The decrease in plasma and colonic GPx activity observed in the loperamide group compared to the negative control was significantly improved by treatment with ZOAE in the last 2 doses (Figure 7E). The ameliorative effect of ZOAE for SOD activity was observed at the highest dose with an increase of 23.60% and 26.85%, respectively, for the colon and the plasma compared to the loperamide group (Figure 7F).

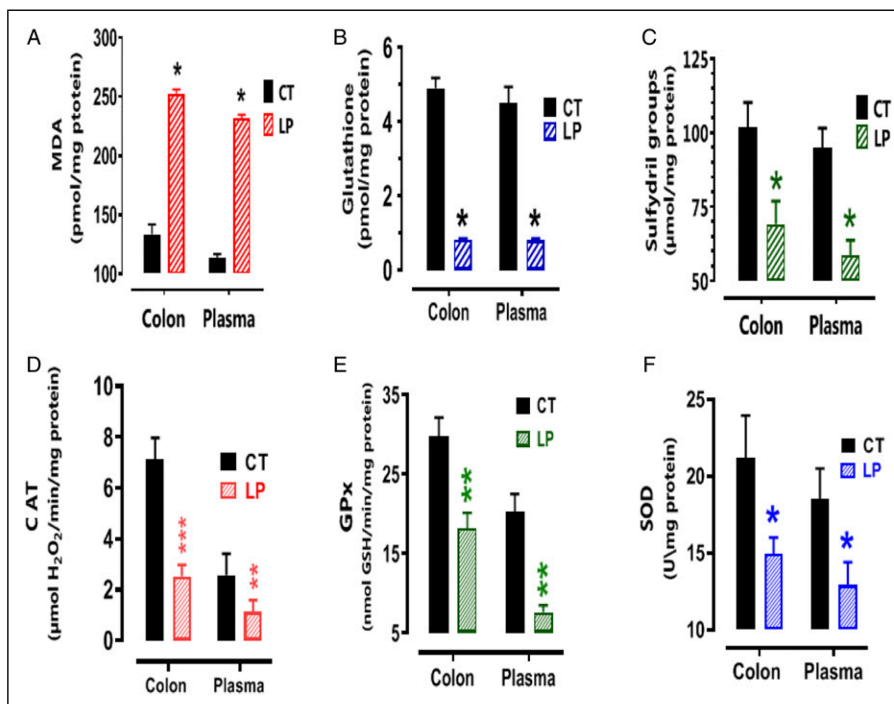

Figure 5. Effect of LP on colon and plasma MDA concentration (**A**), reduced glutathione (**B**), sulfhydryl groups (**C**), catalase (**D**), glutathione peroxidase (**E**), and superoxide dismutase (**F**) activity after LP-induced constipation in a rat model. Data are expressed as means ± SEM (n = 6). *: $P < .05$ compared with the no treated rats (ANOVA test).

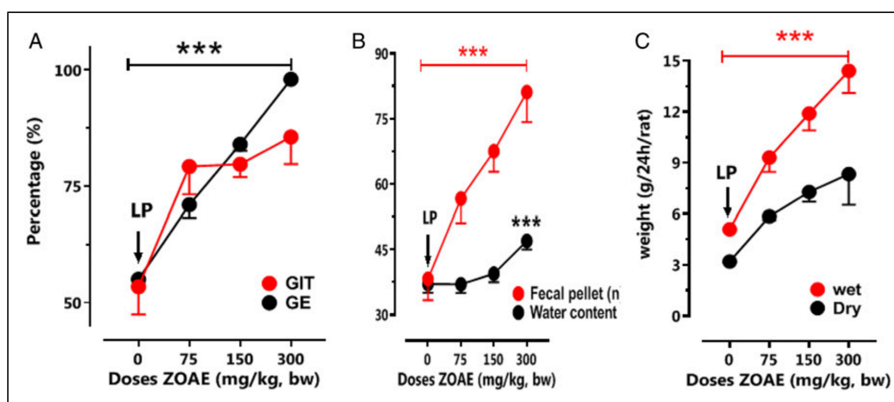

Figure 6. Effect of ZOAE on GIT and GE (**A**), as well as its action on total number, water content of fecal pellet (**B**), and wet/dry fecal weight (**C**) after LP-induced constipation in a rat model. Animals were treated with different doses of ZOAE (75, 150, and 300 mg/kg, b.w.) 1 hour after LP-administration. Data are expressed as means ± SEM (n = 6). *: $P < .05$ in comparison with the no treated group (ANOVA test).

Effect of ZOAE on the Histology of the Colonic Mucosa

Colonic tissues were histologically studied to verify whether LP could alter the morphology of the mucous layer H&E staining showed that the structure of the colonic mucosa is conserved in the negative control group (Figure 8A). On the other hand, the treatment with LP presents a shorter layer of the mucosa compared to normal rats and induces an infiltration of inflammatory cells in the mucous layer (Figure 8A and B). The administration of ZOAE could protect the colon by maintaining the thickness of the mucosa and reducing the inflammatory infiltrate compared to the constipated group and the YOH group where the infiltration of inflammatory cells into the mucous layer is observed more abundant (Figure 8A and E). This protective effect is more marked at the 2 highest concentrations (Figure 8A, C, and D).

Sections stained with Alcian blue were examined for mucus detection. LP-induced a decrease in mucus secretion (Figure 8B) compared to the negative control group where the secretion is observed normal (Figure 8A and B). Treatment with ZOAE

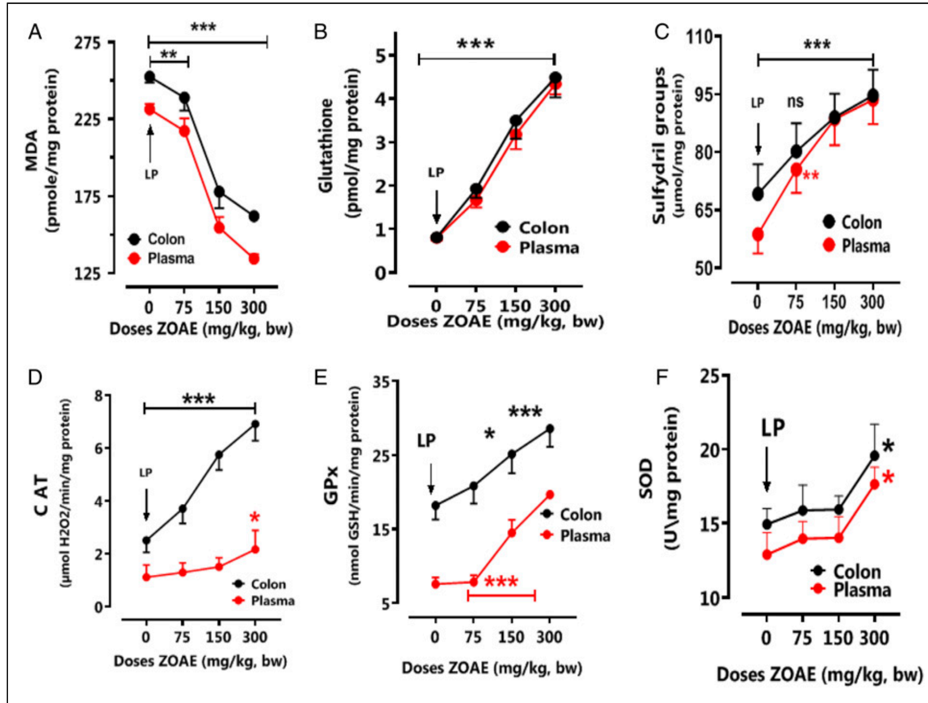

Figure 7. Reverse action of ZOAE on oxidative stress indicators [MDA (**A**), reduced glutathione (**B**), sulfhydryl groups (**C**), catalase (**D**), glutathione peroxidase (**E**), and superoxide dismutase (**F**)] after LP-caused constipated rat model. Animals were treated with different doses of ZOAE (75, 150, and 300 mg/kg, b.w.) 1 hour after LP-administration. Data are expressed as means ± SEM (n = 6). *: $P < .05$ in comparison with the no treated group (ANOVA test).

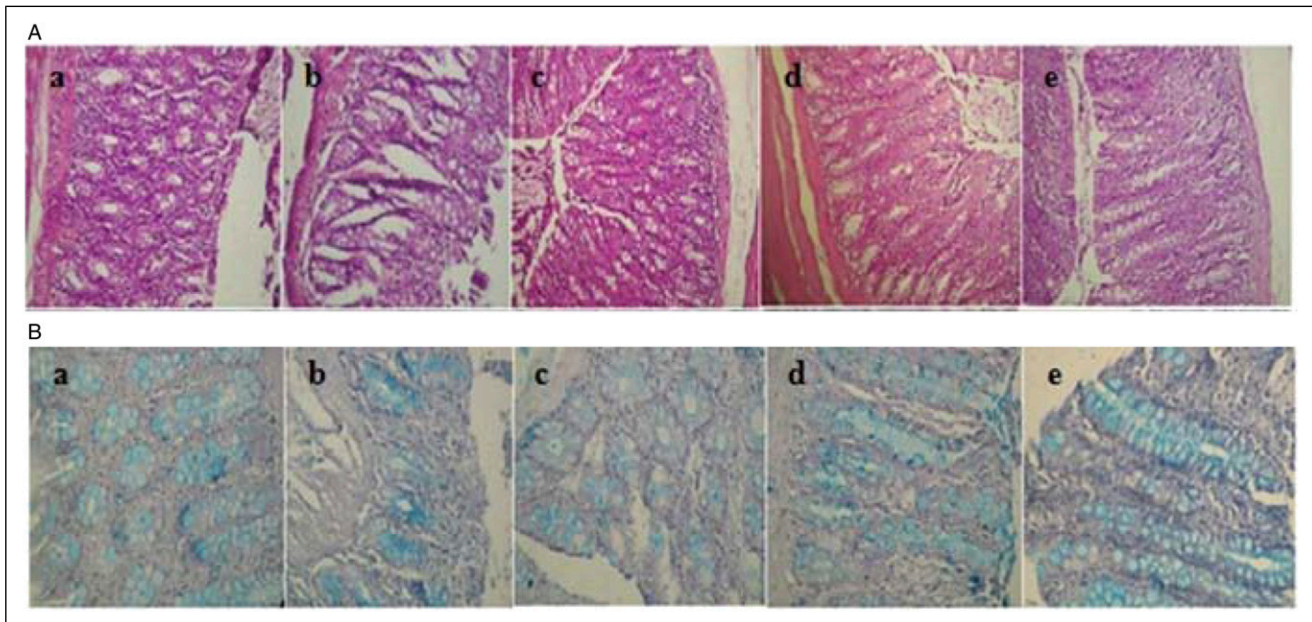

Figure 8. Effect of ZOAE on colon mucosa structure and production of the mucus during LP-induced constipation. Animals were administrated with different concentrations of ZO (75, 150, and 300 mg/kg, b.w.), YOH (2 mg/kg, b.w.), and NaCl solution .9% 1 h after LP administration. Constipated rats (LP) were treated only with LP molecule (3 mg/kg, b.w.). (**A**) Negative control, (**B**) constipated group, (**C**) LP + ZOAE 150 mg/kg, b.w., (**D**) LP + ZOAE 300 mg/kg, b.w., and (**E**) LP + YOH. The histopathological modifications in the slide portions of colon tissue were analyzed by staining with H&E followed by monitoring at 40x.

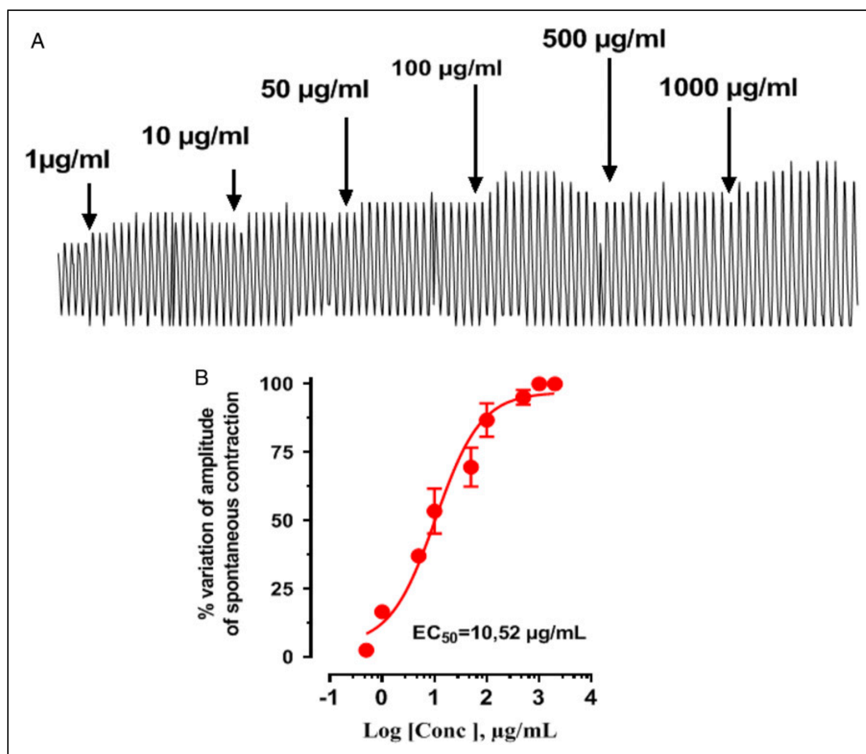

Figure 9. Typical recording of the effect of ZOAE on spontaneous contraction of mouse jejunum (**A**). The concentration–response effect of ZOAE at concentrations of 1–1000 μg/mL (**B**). This figure shows that the concentration of ZOAE which induces 50% of the maximum concentration (EC50) is 10.520 μg/mL. The concentration–response curve was obtained using nonlinear regression using Hill's equation by an iterative least-squares method.

increased the amount of mucus associated with an improvement of producing cells compared to other groups (Figure 8B, C, and D).

Effect of Zingiber officinale *Aqueous Extract on Spontaneous Contraction of the Intestine*

The results obtained in Figure 9 showed that the ZOAE does not induce intestinal relaxation or contraction. However, ZOAE induces an increase of the amplitude of spontaneous contraction of the intestine as a function of various doses (1, 10, 50, 100, 500, and 1000 μg/mL) with EC_{50} of 10.52 μg/mL.

Effect of Zingiber officinale *Aqueous Extract on the Nervous System*

Figure 10A shows that the ZOAE does not inhibit the contraction induced by CarbCh which activates acetylcholine receptors as a cholinergic agonist. In addition, Figure 10B and C show that ZOAE when used before CarbCh rather reduces the frequency and causes an increase in the contraction induced by CarbCh. ZOAE is a neurotrope and it acts weakly on the nervous system.

Effect of Zingiber officinale *Aqueous Extract on Smooth Muscles*

The results in Figure 11 showed that the ZOAE at a concentration of .5 mg/mL does not reduce the intestinal contraction induced neither with 25 mM of KCl nor that induced by 10 mM of $CaCl_2$, so it did not have direct effect on smooth muscles, which makes it possible to deduce that the ZOAE is not a musculotrope.

Effect of Zingiber officinale *Aqueous Extract on Fluid Secretion (Using Chamber)*

Figure 12 showed that the ZOAE has no effect on the short circuit current (*Isc*). Likewise, this extract has no effect on the increase in short circuit enhanced by an adenylate cyclase activator or forskolin (FSK, 10 mM, serosal addition). ZOAE does not influence the intestinal secretion of water and electrolytes caused by FSK which excludes its effects as a secretagogue and an anti-secretagogue.

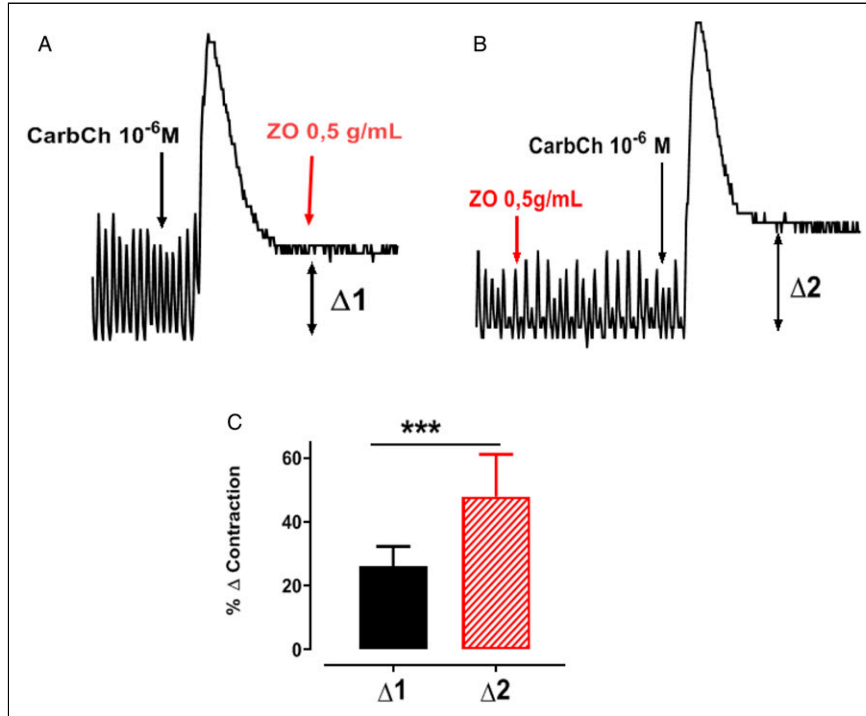

Figure 10. Effect of ZOAE (500 µg/mL) on the contraction induced by CarbCh (10^{-6} M) before (**A**) and after (**B**) its stimulation.

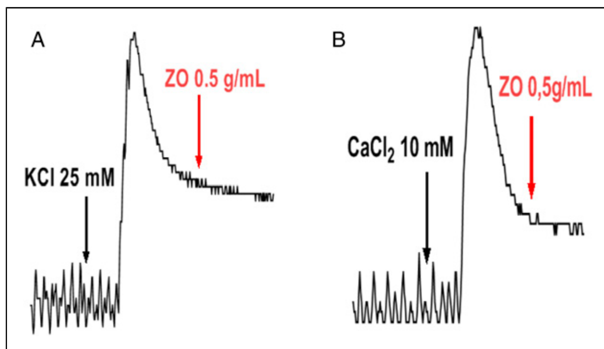

Figure 11. Effect of ZOAE on intestinal contraction induced by 25 mM KCl (**A**) and 10 mM $CaCl_2$ (**B**).

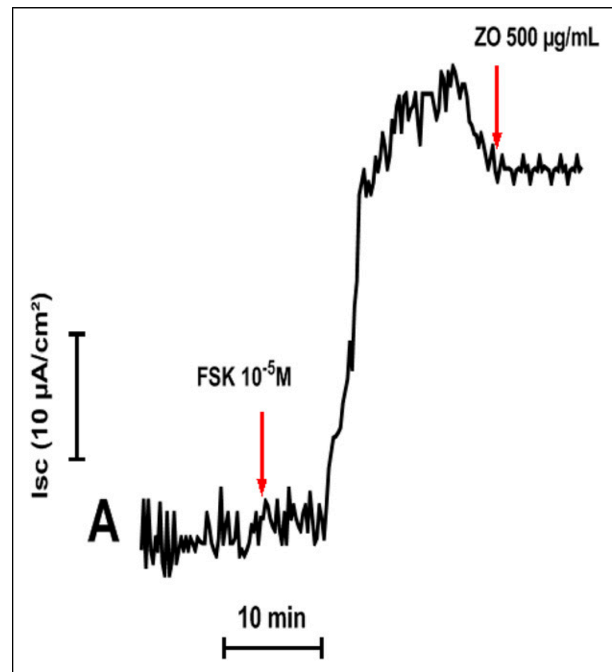

Figure 12. Typical short circuit current recording after addition of FSK 10^{-5} M and ZOAE (500 µg/mL).

Discussion

In the recent research, the laxative/purgative actions of ZOAE were assessed based on various modifications in numerous parameters such as fecal signs (numbers, weight, and water content), GI-motility, spontaneous intestinal contraction, intestinal water and electrolyte absorption/secretion processes, and the thickness of the colonic mucous in vivo after LP-produced a slowing of frequent colonic transit, a rat constipation model. These ZOAE-actions were compared with those obtained with the YOH, an alpha-2 adrenergic antagonist, as a reference drug.

Firstly, the data demonstrated that phytochemical analysis of ZOAE using LC-HRESIMS technique reported the existence of 11 phenol carboxylic acids and 12 flavonoids. Similar results showed the presence of some phenolic compounds obtained in this study.[37] The medicinal use of these compounds may be related to their antioxidant abilities. In this

context, many studies have shown that phenolic acids such as quinic acid, caffeic acid, rosmarinic acid, *trans*-ferulic acid, and *p*-coumaric acid have a strong antioxidant power.[38-40] Equally for flavonoids such as kaempferol, luteolin and quercetin can decrease the oxidative damage induced by reactive oxygen species.[40-42]

It is well-intentioned that the antioxidant capacity of these constituents in natural products is due to their ability to provide electrons or hydrogen atom transfer to scavenge free radicals.[40] The anti-radical DPPH test to confirm the potentiality of the bioactive components of ZOAE to act as donors of hydrogen atoms. The results obtained showed that ZOAE has a high antioxidant power with IC_{50} = .35 ± .01 mg/mL compared to different ginger extracts obtained using four solvents (ethanol, methanol, acetone, and ethyl acetate) randomly collected from local markets of Ayikel and Mandura town, Ethiopia.[43] The inhibitory effect of ZOAE on this free radical can be caused by the polysaccharides of ginger. In this context, many studies have shown that the polysaccharides of *Zingiber officinale* have a high antioxidant potential in vitro by reducing the DPPH radical, hydroxyl radical, and superoxide radical.[44] But this antioxidant power can vary according to the method and the solvent of extraction.[45,46]

The antioxidant activities of the natural product extracts may be resulted from their chelating activity ability against transition metal ions, in particular ferrous and copper ions. This effect seems to be specifically influential for pathogenesis in which immense amounts of metal ions may provoke an oxidation of biological macromolecules especially proteins and lipids.

For this purpose, the chelating capacity of ginger was studied. Indeed, the aqueous extract of ginger showed a weak chelating effect towards Fe^{2+} ions, which can be explained by the existence of small quantities of water-soluble molecules with the capacity to bind or bond with iron ions. In contrast, some studies found a significant chelating activity of the ginger aqueous/ethanolic extracts with a chelating power of 27.3 and 36.2%, respectively, at a dose of 10 µg/mL concentration.[37] And others have shown the same effect for the organic extract of ginger[47] and for the chloroform extract of a species of the *Zingiberaceae* family.[48] These authors reported that structures containing 2 or more of the following functional groups OH, SH, COOH, PO_3H_2, CO, and NR_2 in a favorable functional structure configuration are responsible for the chelating activity.

The acute toxicity assessment showed that ZOAE showed no indicators of toxicity or mortality pending the checking duration with an LD50 higher than the limit dose studied (LD50> 3200 mg/kg).

In addition, to study the gastrointestinal empty stomach, we evaluated the movement of phenol red which is attributable to peristaltic propulsion by monitoring the rate of gastric emptying. The ZOAE accelerates GE-time in a dose-dependent manner compared to the LP-group. These actions are principally due to the existence of the main components in ginger citing gingerols and shogaol and to their activity on muscarinic acetylcholine receptors and 5-HT serotonergic receptors. Pertz et al[49] demonstrated that ginger could act on the 5-HT receptor ion channel complex by blinding the serotonin binding site and Sharma et al[50] reported that ginger inhibits cisplatin-induced delayed gastric emptying. Similar results have shown that *Zingiber officinale* stimulated this digestive phenomenon and antral contractions in patients with functional dyspepsia.[51]

Consumption of ZOAE effectively improved bowel movement, increased the amplitude of spontaneous contraction of the intestine, and elevated stool output. In fact, ZOAE protects against LP-induced constipation by accelerating colonic transit. This protection has also been proven by the increased number of stools and fecal water content compared to constipated rats. Indeed, LP binds to the opiate receptors of intestinal wall cell, inhibits intestinal secretion, slows peristalsis by increasing the time of intestinal transit, and stimulates the electron neutral absorption of water and electrolytes in the enterocyte.[52-54] The laxative effect of ginger can be explained by its high content of carbohydrates and dietary fiber. Ginger contains around 60–70% carbohydrates and 3–8% of total fiber.[55] Via mechanical stimulation the insoluble fibers may induce the intestinal mucosa to mucus water secretion and soluble fibers retain a massive water retention capability to maintain the gut hydration.[56] They must be resistant to fermentation to remain intact and increase the fecal water content. Fibers therefore make the stools denser, more voluminous, and make them retain more water which promotes natural peristalsis and therefore their progression. For these reasons, fibers accelerate colonic motility.

The laxative effect of ZOAE can also be confirmed by its low content of condensed tannins (.33 mg/g DM). It is known that tannins with proteins may form protein–tannate complex and therefore cause their denaturation, which makes the intestinal mucosa higher resistant and decreases water/electrolyte secretion, thus promoting the inhibition of GIT.[57]

GI-hormones modulation such as cholecystokinin (CCK), gastrin (GAS), somatostatin (SS), and motilin (MTL) may also be another influent mechanism for boosting the LOP-induced constipation symptoms in animal models after ZOAE consumption. A recent study revealed that naringenin, a natural flavonoid, regulated the production levels of GI-metabolic components, such as MTL, GAS, endothelin (ET), substance P (SP), acetylcholinesterase (AChE), and vasoactive intestinal peptide (VIP) in serum.[58]

Added to that, it has been shown in many studies that the involvement of the cell types found in the intestinal circular muscle (interstitial cells of Cajal, ICCs) to regular GI-function by production of electrical slow waves and mediating neuromuscular signaling. Injuries to ICCs have been elucidated in several GI motility disorders including constipation. Therefore, we think that ZOAE may excite the membrane potentials by its depolarization. This stimulation can be led to smooth muscle cells through the gap junction. ICCs might act in

response to this membrane depolarization with stimulating effect of the voltage-dependent channels of the calcium ions. Thus, this action of pacemaker potentiality depolarization could produce a GI-motility increase, as with diverse medicinal plants like *Liriope platyphylla* and *Citrus unshiu.*[59]

Decreased mucus generation in the mucosa of the colon is related with colonic slow transit and noted to reduce the colonic mucosal layer thickness, and the number of mucus-generating cells has been investigated by histopathological diagnosis in the constipated animals. In this context, in the treatment of animals with LP for a week, an alteration in the colon microscopical structure was detected in the present study. It not only caused a decrease in the thickness of the mucous layer and the number of mucus-producing cells but also caused inflammation of the colon. This side effect has been proven by several studies. A reduction in mucosal thickness,[59-62] an infiltration of inflammatory cells into the damaged mucosa,[60-63] and a decrease in mucus production were detected in treated rats with LP.[62,63] Mucus is a viscous glandular secretion produced by the mucous membrane and which lubricates the surface of the epithelia, playing a role of protection against microorganisms and harmful substances.[65] Damage to mucus may be caused by reduction in the area of the mucous membrane, and inflammation may be caused by loss of epithelial integrity and decrease in mucus-producing cells.[64,65]

Treatment with ZOAE protects the colon from inflammation that YOH cannot reduce. In this context, many authors have confirmed the effect of this extract against inflammation[66] and others have shown the efficacy of its specific phytochemicals such as gingerol against acute ulcerative colitis.[67] In a dose-dependent manner, ZOAE improves the epithelial integrity by maintaining the thickness of the mucosa and protecting it by the excessive secretion of mucus. This protective effect against structural damage to the colon may be due to the antioxidant properties of certain phenolic compounds in ginger such as quercetin glucoside and caffeoylquinic acid.[68] Similar results have shown that ZOAE increases the content of gastric mucin depending on the dose.[69]

Oxidative stress is a state of lack of balance between the overproduction of free radicals and cellular antioxidant capacities.[70] It is involved in various GI-disorders characterized by disturbance of peristalsis and irregularity in the secretion/absorption process.[71] In this context, Zhou et al[72] reported that chronic constipation, a GI disease, can cause potential oxidative stress in children. Long-term oxidative stress can damage major cellular constituents through the generation of reactive oxygen species (ROS) which may be the subjects of potential injury to DNA, proteins, and lipids.[70,73]

In this study, the inhibition of GI-motility and intestinal secretion induced by LP is accompanied by the increase in plasma and colonic lipoperoxidation and the attenuation of enzymatic (CAT, SOD, and GPx) and non-enzymatic (sulfhydryl group and GSH) antioxidant activities. Our results are close to that of Jabri et al[7] and Sebai et al[62] who proved that LP increases the level of MDA and decreases antioxidant activity.

Treatment with ZOAE restored any oxidative disturbance obtained. It significantly reduced lipid peroxidation thanks to the abundance polyphenolic components as flavonoids like quercetin, kaempferol, apigenin, and luteolin. The latter is capable of reducing free radical chains through electron and proton transfer and chelating transition metal ions capable of catalyzing lipid peroxidation.[74] Our results corroborate with the work of Oboh et al[75] who revealed that the aqueous extract of 2 varieties of ginger (red and white) produced significant reduction in brain MDA in a dose dependent manner and this is due to the fact that phytochemicals compounds in ginger protect against lipid peroxidation by their potential to chelate Fe^{+2} ions and trap hydroxyl radical. Other studies have found that ginger lowers MDA levels and increases plasma antioxidant capacity in diabetic rats.[76]

In addition, the administration of ZOAE at different doses significantly normalizes the activity of non-enzymatic antioxidants, but for the improvement of the activity of enzymatic antioxidants, it is not significant. The bioactive molecules present in ginger can exert their antioxidant activity by increasing the concentration of biologically important endogenous antioxidants.[77] In this context, the antioxidant potential of caffeic acid and ferulic acid has been expressed through their strong iron-reducing activities.[37] They strengthen oxidative defense by increasing levels of endogenous antioxidant enzymes.[78,79] Li et al[80] have also shown that treatment with cinnamic acid increases CAT, SOD, GPx, and GSH activity. As well, rosmarinic acid and *p*-coumaric acid regulate oxidative damage by increasing the activity of these antioxidants.[81,82] The obtained results are in accordance with those found by Abd-Allah et al[83] which provide that ginger elevates the level of reduced glutathione and antioxidant enzymes and declines the MDA level in the intestinal mucosa.

Conclusion

ZOAE promotes GI and colonic motilities by enhancing a laxative action and alleviating oxidative damage, which proved that ZOAE has a strong ability to reduce and prevent slow colonic movements. These data may provide important information for the future researches to elaborate the appropriate underlying mechanisms, the mediated molecular pathways, and the principal bioactive compounds of ZOAE. Thus, the obtained findings suggest the potential of ginger extract as an additive in the food and pharmaceutical industries.

Appendix

Abbreviations

CarbChol	Carbamylcholine
EC_{50}	Effective concentration
FGDs	Functional gastrointestinal disorders
FSK	Forskolin

GE	Gastric emptying
GIT	Gastrointestinal transit
IC_{50}	Inhibition concentration
Isc	Short circuit current
LD50	Lethal dose 50
LP	Loperamide
ROS	Reactive oxygen species
YOH	Yohimbine
ZOAE	*Zingiber officinale* aqueous extract

Acknowledgments

The authors would like to thank all members of TBC Laboratory (Faculty of Pharmacy, University of Lille) for assistance and helpful discussion.

Author Contributions

Conceptualization: H.S., K.R., C.A., and B.E; methodology and data curation: H.S., K.R., C.A., B.E., and B.G; writing-original draft preparation: H.S., K.R., and C.A., writing-review and editing: B.G. and B.E; and supervision and validation: H.S., B.E., and B.G. All authors have read and agreed to the published version of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Tunisian Ministry of Higher Education and Scientific Research.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board at the University of Jendouba, Tunisia.

Ethical Consideration

All procedures on animals in this study were compiled with the National Institute of Health recommendations for the use and care of animals.

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