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Antidiarrheal and Spasmolytic Activity of *Calendula arvensis* Linn in Laboratory Animal Models: The Potential Role of Calcium Channel Blockade

Hesham El Zorba[1], Dina I. A. Derbala[1], Etmad M. Mancy[2], Samy A. F. Morad[3*], Noha Abdelmageed[4*]

[1]Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, [2]Department of Pharmacology, Faculty of Veterinary Medicine, Aswan University, Aswan, [3]Department of Pharmacology, Faculty of Veterinary Medicine, South Valley University, Qena, 83523, Egypt, [4]Department of Pharmacology, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt.

Abstract

Despite significant advancements in the pharmaceutical industry, herbal medicine continues to be the most prevalent form of treatment globally. Traditional plant-based remedies maintain their popularity and widespread use, even as modern medical technologies and synthetic drugs have become increasingly sophisticated. The current research aimed to assess the toxicological characteristics, as well as the antidiarrheal and spasmolytic properties of the hydroalcoholic extract from *Calendula arvensis* Linn (Cal) flowers. The toxicity study revealed LD_{50} of Cal extracts (CalE) was 2450 mg/kg. Utilizing castor oil-induced diarrhea models and enterpooling assessment revealed that CalE has a potent antidiarrheal effect. Employing the "Gastrointestinal motility test" using a charcoal meal confirms the reduction of intestinal peristaltic movement. Oscillograph studies revealed that CalE inhibits intestinal motility in a concentration-dependent manner. CalE did not act on the muscarinic receptors or nicotinic receptors. CalE did not act on both α and β adrenergic receptors as it has no effect on the duodenum in the presence of α adrenergic blocker (Prazosin) and α and β adrenergic receptor blocker (Propranolol). Interestingly, in the presence of $CaCl_2$, CalE exerted an inhibitory effect on the motility of the duodenum, similar to the effect of verapamil and in dose-dependent manner. Thus, this data indicates that CalE exhibits antidiarrheal and spasmolytic impacts by blocking calcium channel.

Keywords:
Antidiarrheal, *Calendula arvensis* Linn, Calcium channels, Spasmolytic activity, Toxicological study.

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*Corresponding Authors: Noha Abdelmageed E-mail: nohafoud@gmail.com,
Samy Morad, E-mail: samymorad@gmail.com
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Competing interest: The authors have declared that no competing interest exists.

Introduction

Throughout history, humans have relied on medicinal plants to treat a wide range of ailments affecting both people and animals. Even in modern times, plant-based therapies remain the most prevalent form of medicine globally. The effectiveness of these natural remedies can be attributed to the presence of specific bioactive compounds in medicinal and aromatic plants. These compounds include saponins, tannins, essential oils, flavonoids, alkaloids, and various other chemical substances, each contributing to the plants' therapeutic properties. The diverse array of active ingredients found in medicinal plants continues to play a crucial role in traditional and alternative medicine practices worldwide. (Abdelmageed et al., 2017, Abdelmageed et al., 2023) . There is a growing interest in natural products within the medical field (Abdelmageed et al., 2021, El Gaafary et al., 2022, Morad and Cabot, 2013, Morad et al., 2016), and their therapeutic benefits is gradually increasing , in comparison to synthetic drugs that have severe side effects. Therefore, a better therapeutic approach should be established to discover a novel natural bioactive compound with potent therapeutic activities and less side effects. considerable focus has been directed towards exploring the potential health-enhancing attributes of phenolic compounds (Yen et al., 2018, Tungmunnithum et al., 2018, Gunes-Bayir et al., 2018, Telles et al., 2017, GutiErrez-Grijalva et al., 2016, Kartal, 2007). *Calendula arvensis Linn*, is an annual herbaceous Euro-Mediterranean species belonging to the Asteraceae family, was extensively cultivated by ancient civilizations such as the Egyptians, Greeks, Hindus, and Arabs (NGRP, 2012, USDA, 2016), and The blossoms of this plant are employed in diverse pharmaceutical formulations, primarily in the form of ointments, to address an array of dermatological ailments. These conditions encompass wounds, ulcers, eczema, bruises, burns, eruptions, varicose veins, and cutaneous hemorrhoids. (Zitterl-Eglseer et al., 1997, Arora et al., 2013, Tiwari, 2008). Flower preparations have also many other therapeutic uses; choleretic, anti-inflammatory, analgesic-antipyretic, disinfectant-antibacterial, anti-cancer, , sedative, diuretic, and tonic actions (Duke, 2002, Arora et al., 2013) . Traditionally, in Egypt, *Calendula arvensis* L (Common names: *Calendula aegyptiaca* Pers, Field marigold, Asteraceae) (NGRP, 2012) has been used for treatment of constipation and abdominal cramps, although the underline mechanism is still unclear. Therefore, the present study aims to investigate toxicological activity, antidiarrheal and spasmolytic activity of Cal 70% ethanol extract (CalE) and determine the possible underline mechanism of action.

Materials and Methods

Drugs

The chemical compounds, namely Acetylcholine (ACh), Propranolol, Prazosin, verapamil, nicotine sulfate, and calcium chloride, were procured from Sigma Aldrich, an esteemed supplier based in St. Louis, USA. Prior to their usage, all drugs were prepared by dissolving them in DMSO (dimethyl sulfoxide) and subsequently diluted in Tyrode's solution.

Plant

Cal-flowers were collected and identified at the Faculty of Pharmacy-Cairo University, with a designated voucher number CUVM2015-V3. Subsequently, the flowers underwent a series of procedures including air drying, pulverization, and storage in a securely sealed glass container. These steps were taken to facilitate subsequent investigations in the fields of phytochemistry, toxicology, and pharmacology.

Preparation of Cal-flower extract

The extraction process involved the utilization of 70% ethanol, through which dried Cal-flowers powder was percolated multiple times until complete exhaustion (Hesham et al., 2016), Subsequently, the extracted solution was subjected to filtration using a paper filter. Most of the solvent was subsequently eliminated through employment of a Rotatory evaporator, employing a low temperature of 50 ºC.

Animals

Mature mice and rats of both sexes weighing 20-25 gm and 160-190 gm, respectively, were used in this study. Adult white albino rabbits 1-2 kg were used for oscillograph studies. The animals were kept and maintained under standard laboratory conditions of temperature, humidity, and light—all experiments conducted after 2 weeks (adaptation period) of animal arrival to the lab. This study has been approved by the Institutional Animal Ethical Committee, under registration number 2019/CU/ FVM/4.

Preparation of organ bath

The study involved an examination of the motility of the duodenum of an isolated rabbit using a glass jar bath apparatus. The apparatus consisted primarily of a glass water bath with a capacity of approximately 750 mL, which was placed within a metal stand. This stand contained a portable electric heater that was utilized to maintain the water temperature at a constant 37 C. An inner glass tube, referred to as the organ bath, with a capacity of around 50 mL, extended through the bottom of the stand. This organ bath was connected to an inverted T-shaped glass tube. One branch of the T-shaped glass tube was linked to a rubber tubing equipped with a clamp, which served the purpose of draining the solution. The other branch of the T-shaped glass tube was connected to a bottle positioned at a higher level, containing fresh Tyrode's solution. Within this organ bath, a glass cannula, serving as an oxygen tube, was securely held in place using a clamp attached to one of the two upright stands. On the other hand, a T2 isotonic transducer was connected to the apparatus, which was then linked to a two-channel oscillograph MD2. To prepare the apparatus for experimentation, the temperature of the outer bath was adjusted to 37 C. The organ bath was filled with Tyrode's solution, and oxygen was allowed to pass through it.

Experimental design:

Acute-Toxicological study and determination of LD50:

Preliminary investigations were conducted to ascertain the LD50 of CaL extract, using the methodology previously outlined by Kerber in 1941 (Kerber, 1941). To achieve this objective, five groups, each consisting of five mice weighing between 20-25 gm, were administered intraperitoneal injections of increasing

doses of the extract, ranging from 1500 to 3500 mg/Kg body weight. The control group received only the diluent. Following a 24-hour observation period, the toxic symptoms, mortality rate, and post-mortem findings were documented for each group. The LD_{50} of the tested extract was then calculated using the formula provided below:

The formula

$$LD50 = {}^{(\sum AxB)}/_{N}$$

This formula represents a quantitative measure used in toxicology studies to determine the lethal dose at which 50% of a population of animals will succumb. In this equation, several variables are considered. Firstly, DM refers to the maximum dose required to cause mortality in all animals tested. Additionally, the variables A and B represent the mean number of died animals between two consecutive groups and the difference in dose between these groups, respectively. Finally, N denotes the number of animals included in each group. The symbol $\sum$ signifies the summation of the products obtained by multiplying A and B.

Assessment of Antidiarrheal effect of Calendula arvensis L extract

Castor oil-induced diarrhea-model

Albino Swiss rats (120-160 g, either sex) were allocated into 6 groups (6 rats in each group). The animals were fasted overnight (18 hrs). Free access to water was allowed before the Caster oil was administered to all groups of animals. Diarrhea was induced by oral administration of 1 mL of castor oil. One hour before castor oil administration, the control animal group (group-1) received only 2 mL/kg of normal saline, Group-2 (standard group) received atropine (3 mg/kg, i.p), as a standard drug; Groups 3, 4 and 5 received various concentration CalE (200, 400 and 600 /kg, respectively). Each animal was housed in an individual-cage, featuring a flooring covered with blotting paper that was replaced on an hourly basis. The number of diarrheal droppings was counted for 4 hr. Stools passed by the CalE-treated groups were compared with the standard (castor oil)-treated group. Data presented as the number of defecation and percentage presentation. The percentage of diarrheal inhibition was calculated according to the following equation: Percent (%) inhibition = [(T1-E)/ T1] ×100; The variable denoted as T1 represents the average number of defecation occurrences induced by the administration of castor oil. On the other hand, the variable E signifies the average number of defecation occurrences resulting from the administration of the extract.

Gastrointestinal motility test (Charcoal meal intestinal transit test)

Rats (30 rats) fasted for 18-hrs and were divided into five groups of six animals each. Group-1 (negative control) animals served as the control, treated orally with distilled water. Group-2 animals served as positive control and were treated with atropine (3 mg/kg, i.p.). Animals in 3rd, 4th and 5th groups were received orally 200, 400, and 600 mg/kg of CalE. One hour later, each animal was administered 0.25 mL of a charcoal meal (10 % charcoal in distilled water, orally). After 30 min, the animals were sacrificed. The total length of the small intestine, from the pylorus to caecum, and the length traveled by the charcoal meal were measured and recorded. The results were expressed in terms of "charcoal-travel

distance" in cm and as a percentage of the length of the small intestine (movement, %) in order to make a meaningful comparison.

Castor oil-induced enteropooling method

Castor oil-induced enteropooling (intestinal fluid accumulation) was used to evaluate the antisecretory activity of CalE. The tested rats were fasted for 18 hrs before conducting the experiment. The animals in the positive control group received atropine sulfate (3 mg/kg, intraperitoneal injection, i.p), while those negative control groups received only distilled water (1 mL, orally). Rats within the experimental groups were administered a uniform quantity of CalE, which corresponded to specific dosages of 200, 400, and 600 mg/kg, respectively. After 1 hr, all animals received orally castor oil (1 mL). One hour later, all rats were sacrificed as described and the small intestine was excised, tied from both ends and weighted, then the intestinal contents were squeezed out into a measuring cylinder to determine the volume and weight of the intestinal content. Data were presented as "intestinal content (gm or mL)" and percentage of positive control.

Assessment of spasmolytic activity of Calendula arvensis L extract

The impact of CalE was examined on the isolated duodenum of rabbits in accordance with the experimental methodology outlined by Dar and Channa (Ahsana Dar and Shaban Channa, 1999)**.** Rabbits were freshly slaughtered, the first part of the intestine (duodenum) was carefully dissected. Intestinal tissue was impregnated with warm, 37 °C, Ringer's solution (137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 2 mM Na_2HPO_4, and 5.6 mM glucose). A duodenal strip measuring approximately one inch in length was affixed within the organ bath apparatus through the attachment of one end to a glass cannula, while the other end was secured using a thread connected to a T2 isotonic transducer. After 15 min of equilibration, the Bioscience MD2 Biographic Recorder was used to record normal intestinal motility. Normal rhythmic contractions of the intestinal strips were first recorded then the effects of graded increased doses of the extract (from 0.01 – 1.00 mg /mL) were recorded.

Explore the mechanism of action involved in spasmolytic activity

Numerous experimental sets were conducted subsequent to the investigation of the typical intestinal motility and the impact of escalating doses of the examined extracts, with the objective of identifying the precise location where CalE exerts its influence. In the first experimental set, we investigated the possibility of involvement of the cholinergic pathway (an atropine-like activity, muscarinic acetylcholine receptor) in the spasmolytic activity of CalE. In this set, we added acetylcholine (ACh) at 5×10^{-7} M concentration after CalE. In contrast, the present study aimed to examine the likelihood of ganglionic blocking activity through the introduction of a minute quantity of nicotine sulfate at a concentration of 2×10^{5} M subsequent to CalE administration. The second experimental set was established to investigate whether the adrenergic pathway is involved in the action CalE. In this set, the α-adrenoceptor agonist activity of the studied extract was tested by blocking the α-receptors with Prazosin at 4×10^{-4} mg/mL bath then the effective dose of the extract

was studied. In contrast, the probability of adrenergic blocking activity was tested by the addition of Propranolol as both α and β adrenergic receptor blocking agent, then the effective dose of extract was added.

The last experimental set was conducted to assess the role of calcium channels in the spasmolytic activity of the CalE. In this set, the probability of blocking calcium channels was tested by adding calcium chloride at a concentration of 5 x 10^{-3} M on the isolated rabbit's duodenum that caused stimulation of the intestinal motility via calcium modulation, then the effective dose of extract was added. Verapamil was used as standard control.

Statistical analysis

The data was presented as mean ± standard deviation (SD) along with a control percentage. To compare the groups, a One-way Analysis of Variance (ANOVA) test was employed, and the p-values were determined using the Tukey post hoc test. Statistical analysis of the data was performed using GraphPad-Prism v7 software. Statistical significance was defined as p-values less than 0.05.

Results

Toxicological study

A study was conducted to investigate the acute toxicity of *Calendula arvensis* Linn extract in mice through intraperitoneal injection. The obtained data was subjected to thorough analysis, which unveiled that the minimum lethal dose of CalE is 2000 mg/kg body weight. Notably, the observed symptoms of toxicity induced by CalE were primarily of neurological nature, including tremors, convulsions, and arched back, accompanied by an accelerated respiratory rate leading to coma and eventual demise. Subsequent post-mortem examination exhibited the presence of minor hepatic petechial hemorrhages as well as static congestion in the lungs, heart, and kidneys. The calculated LD_{50} of CalE was determined to be 2450 mg/kg body weight.

Assessment of Antidiarrheal activity of Calendula arvensis L extract

To evaluate the therapeutic impact of CalE as an antidiarrheal agent, we employed three experimental approaches: (1) Castor oil-induced diarrhea-model, (2) Charcoal meal intestinal transit test, (3) Castor oil-induced enteropooling method.

Castor oil-induced diarrhea-model

As depicted in Fig. 1A, all tested CalE exhibited very significant antidiarrheal activity against castor oil-induced diarrhea. CalE doses (200, 400, 600 mg/kg) significantly decreased ($P < 0.01$) the total number of wet feces (13.60 ± 1.50, 9.8 ± 1.48 and 6.4 ± 1.14, respectively) produced upon oral administration of castor oil in comparison to castor oil-treated group (20.40 ± 3.507), Fig. 1A.

The percentage protection against castor oil-induced diarrhea by 200 mg/kg, 400 mg/kg, 600 mg/kg of CalE, with respect to control, was found to be 29.00 ± 12.45, 51.00 ± 7.41 and 68.00 ± 5.70 respectively (Fig. 1C). CalE at a dose of 600 mg/kg showed higher percentage protection. Both CalE at 400 and 600 mg/kg exhibited antidiarrheal activity similar and non-significant to the standard drug, atropine sulfate, which exhibited a significant reduction of defecation number (6.400 ± 1.140) and produced 68.00 ± 5.701 % protection activity (Fig. 1A-1C).

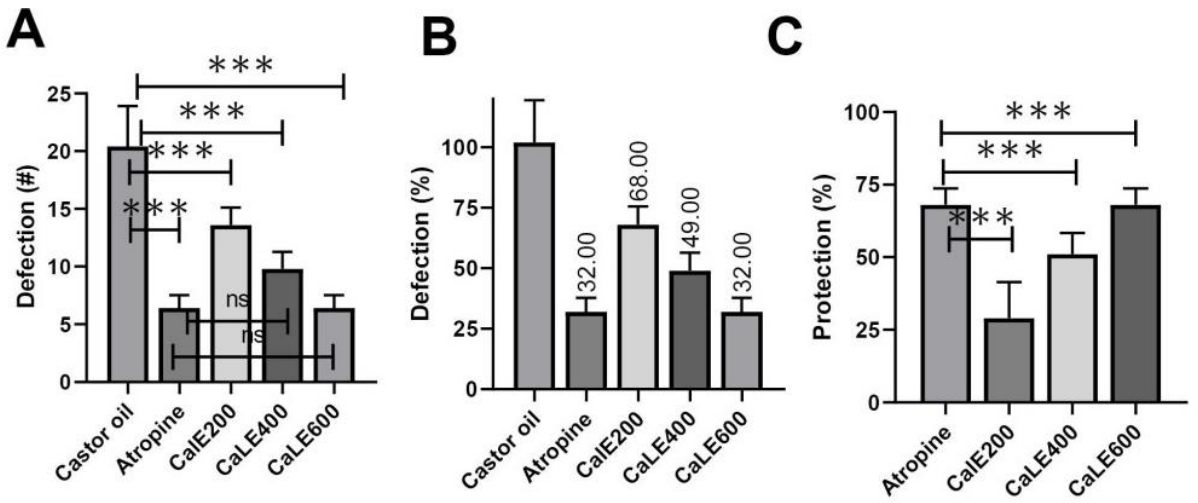

Fig. 1. Evaluation of anti-diarrheal activity of hydroalcoholic extract of *Calendula arvensis* L (CalE) against castor oil-induced diarrhea. **(A)** the number of defecations in rat treated with different substances: Castor Oil, Atropine, and varying concentrations of *Calendula arvensis* L (CalE200, CalE400, CalE600). The asterisks indicate significant differences compared to the control group (Castor Oil). **(B)** The percentage of defecation in rat treated with different substances: Castor Oil, Atropine, and varying concentrations of *Calendula arvensis* L (CalE200, CalE400, CalE600). The asterisks denote statistical significance compared to the control group. **(C)** The protective effect (% protection) of different treatments against castor oil-induced diarrhea in rat. Treatments include Castor Oil, Atropine, and varying concentrations of *Calendula arvensis* L (CalE200, CalE400, CalE600). The asterisks highlight statistically significant differences from the control group (Atropine). Data represents mean ± SD, *** $P < 0.001$.

Gastrointestinal motility test (Charcoal meal intestinal transit test)

Data analysis for the "charcoal meal intestinal transit test" was depicted in Fig. 2A-C, as "travel distance" by cm, "percentage movement" and "percentage of protection". CalE (200, 400, and 600 mg/kg) decreased significantly the impetus of charcoal meal through the gastrointestinal tract (traveling distance, cm, 53.83 ± 8.658, 26.33 ± 2.066 and 12.33 ± 1.75, respectively) when compared with control (84.17 ± 3.43 cm), with highly therapeutic impact comparing to atropine sulfate (49.17± 7.19 cm) (Fig. 2A). Percentage of gastrointestinal movement illustrated in Fig. 2B, where the percentage of protection was calculated and depicted in Fig. 2C. The percentage protection (% of inhibition of movement) of 200 mg/kg, 400 mg/kg, and 600 mg/kg of CalE, with respect to control, was found to be 35.97 ± 10.50, 68.74 ± 1.46, and 85.31 ± 2.32 % respectively (Fig. 2B and 2C). Maximum protection was achieved by both 400 and 600 mg/kg. CalE at a dose of 200 mg/kg did not show any significant difference in the protection compared to atropine (41.32 ± 0.30), where 400 mg and 600 mg/kg showed higher significance ($P < 0.01$) protection than atropine.

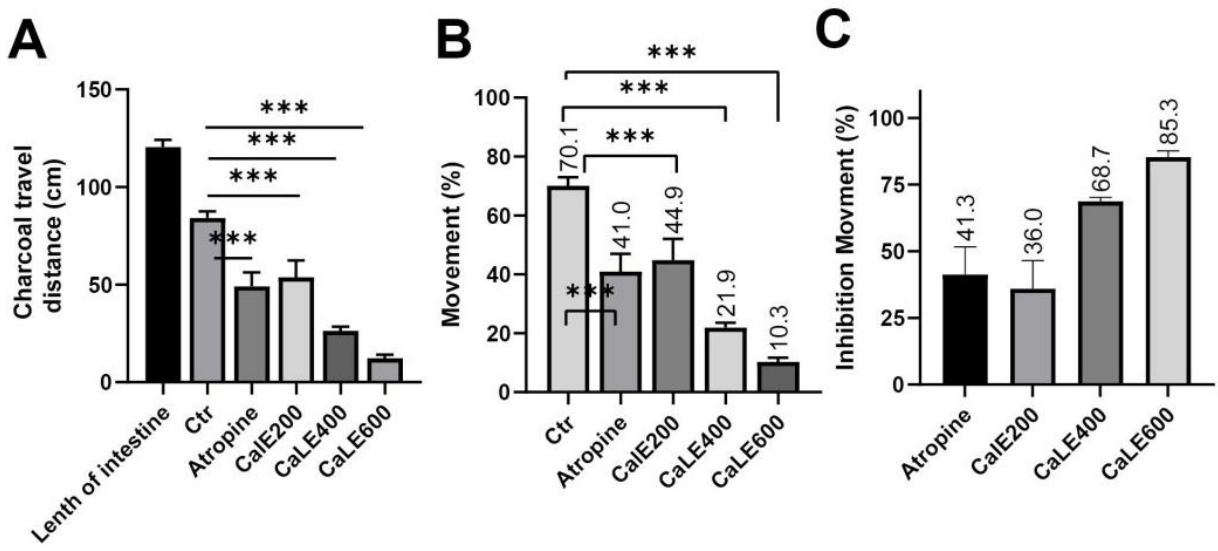

Fig. 2. Evaluation of Anti-Diarrheal Activity of Hydroalcoholic Extract of *Calendula arvensis* L (CalE) in Gastrointestinal motility test. **(A)** Charcoal travel distance: The length of charcoal movement along the intestine was measured to assess motility. Treatment with CalE significantly reduced the travel distance compared to the control group (**P < 0.001). **(B)** Intestinal Movement (%): Percentage inhibition of intestinal movement was calculated relative to the control. CalE treatments at different concentrations showed significant inhibitory effects on intestinal movement (**P < 0.001). **(C)** Inhibition of Intestinal Movement (%): The percentage inhibition of intestinal movement by CalE is presented. Higher concentrations of CalE exhibited greater inhibitory effects (**P < 0.001). Atropine served as a positive control. *** indicates statistical significance with P < 0.001 when compared to the control group. Data represents mean ± SD

Castor oil-induced enteropooling method

CalE showed a significant reduction (P < 0.01) in both average weight and volume of intestinal contents in comparison to the negative control. CalE at doses of 200, 400, 600 mg/kg, exhibited a significant (P < 0.1 and P < 0.01) decrease in intestinal content (0.70 ± 0.12, 0.54 ± 0.054 and 0.52 ± 0.08 gm, respectively) (Fig. 3A-3C), as compared to castor oil-treated animals (1.08 ± 0.29 gm), which represented 70.71 ± 12.37, 54.55 ± 5.53 and 52.53 8.45 % as compared to castor oil-treated animals (Fig. 3B). CalE at 400 and 600 mg exhibited maximum inhibition and showed the same activity, which is similar to loperamide, a standard drug.

The other parameters recorded in this experiment were the intestinal volume (mL) and percentage of intestinal volume in the average volume of intestinal contents. CalE showed a significant reduction

(P < 0.01) in both average volumes of intestinal contents in comparison to the negative control. CalE at doses of 200, 400, 600 mg/kg, exhibited a significant (and P < 0.01) decrease in intestinal content (3.62 ± 0.30, 2.56 ± 0.15 and 1.98 ± 0.32 mL) (Fig. 3D-3F), as compared to castor oil-treated animals (4.64 ± 0.38), which represented 80.44 ± 6.74, 56.89 ± 3.37 and 44.00 ± 7.26 mL (Fig. 3D)

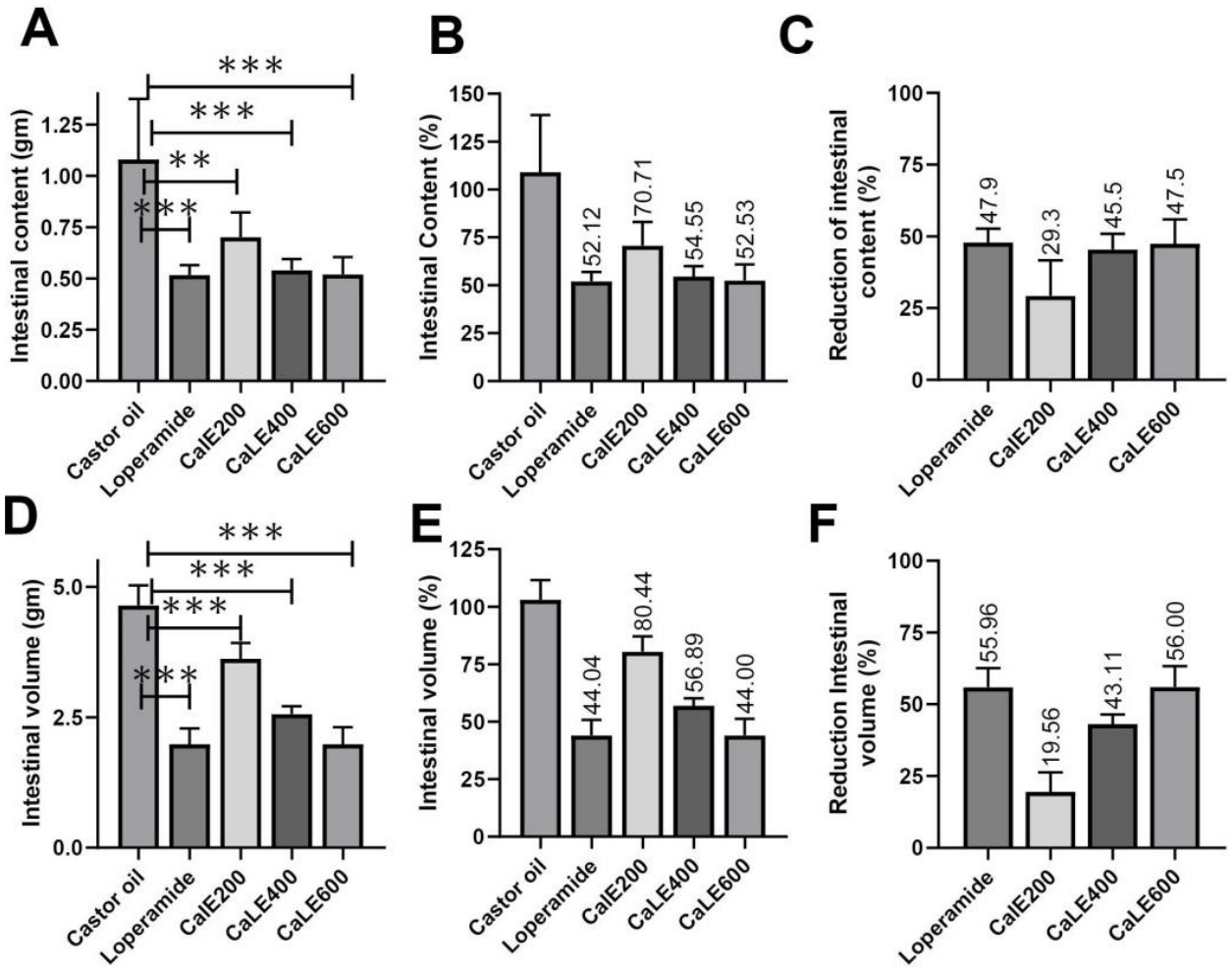

Fig. 3. Evaluation of anti-diarrheal activity of hydroalcoholic extract of *Calendula arvensis* L (CalE) against castor-induced enteropooling. **(A)** Intestinal Content (gm): This panel shows the amount of intestinal content in grams after treatment with castor oil, loperamide, or different doses of CalE (200, 400, and 600 mg/kg). Statistical significance is indicated by asterisks (** $P < 0.01$, *** $P < 0.001$). **(B)** Intestinal Content (%): Here, the percentage of intestinal content relative to the control group is depicted. Significant reductions are observed with increasing doses of CalE. **(C)** Reduction of Intestinal Content (%): This panel quantifies the percentage reduction in intestinal content compared to the control group. Higher doses of CalE show greater reductions. **(D)** Intestinal Volume (gm): The volume of intestinal contents is presented here. Similar trends are observed as in Panel A, with significant reductions noted at higher doses of CalE. **(E)** Intestinal Volume (%): The percentage change in intestinal volume from the control group is shown. There is a clear dose-dependent effect of CalE on reducing intestinal volume. **(F)** Reduction of Intestinal Volume (%): Lastly, this panel illustrates the percentage reduction in intestinal volume compared to the control group. Data represents mean ± SD.

Spasmolytic effect

The effect of CalE on the isolated rabbit's duodenum and the possible underline mechanism are demonstrated in Fig. 4 to Fig. 6. As shown in Fig. 4, CalE at different concentrations of 0.01, 0.1, and 1 mg/mL caused inhibition of the motility of the isolated rabbit's duodenum, in a concentration dependent manner.

To evaluate the role of the parasympathetic system on the spasmolytic activity of CalE, Ach (muscarinic agonist) and nicotine (in a small dose, nicotinic agonist) were employed. The addition of acetylcholine (ACh) at $5x10^{-7}$ M concentration after CalE at a concentration of 0.1 mg/mL bath induced marked stimulation of the motility of the isolated rabbit's duodenum. This trial denotes that CalE did not act on the muscarinic receptors as shown in Fig. 5A. Additionally, the addition of nicotine sulfate (small concentration) at a concentration of $2x10^{-5}$ M after CalE at 0.1 mg/mL also induced a stimulatory effect on the motility of isolated rabbit's duodenum. This denotes that CalE did not act on the nicotinic receptors (Fig. 5B).

To evaluate the role of the sympathetic system on the spasmolytic activity of CalE, Prazosin (alpha (α) adrenergic blocker) and Propranolol (both α and β adrenergic receptor blocking drug) were utilized in this experiment. The addition of CalE after Prazosin at $4 x10^{-4}$ mg/mL did not affect the inhibitory activity of Prazosin on the motility of isolated rabbit's duodenum as shown in Fig. 5C. This excluded the effect of CalE on α adrenergic cell receptors. Moreover, as shown in Fig. 5D, the addition of CalE at a concentration of 0.1 mg/mL after Propranolol, did not affect the inhibitory effect of Propranolol on the motility of isolated rabbit's duodenum. This confirmed that the effect of CalE was not mediated through α or β adrenergic receptors.

Knowing that calcium channels are essential for controlling intestinal motility, we planned to determine whether CalE exerts spasmolytic activity via calcium channels. As shown in Figure 6 A, the addition of calcium chloride ($CaCl_2$) at a concentration of $5x10^{-3}$ M on the isolated rabbit's duodenum caused stimulation of the intestinal motility via calcium modulation. Interestingly, the addition of CalE at a concentration of 0.1 and 0.3 mg/mL, after $CaCl_2$ –mediated intestinal motility, exhibited a concentration-dependent inhibitory effect on the motility of isolated rabbit's duodenum as shown in Fig 6B and Fig 6C, which is similar to verapamil effect (Fig. 6D). This indicates that CalE may produce its inhibitory effect of the isolated rabbit's duodenum through calcium ion modulation. Verapamil (Fig. 6D) was used as a standard drug, and calcium channel blocker.

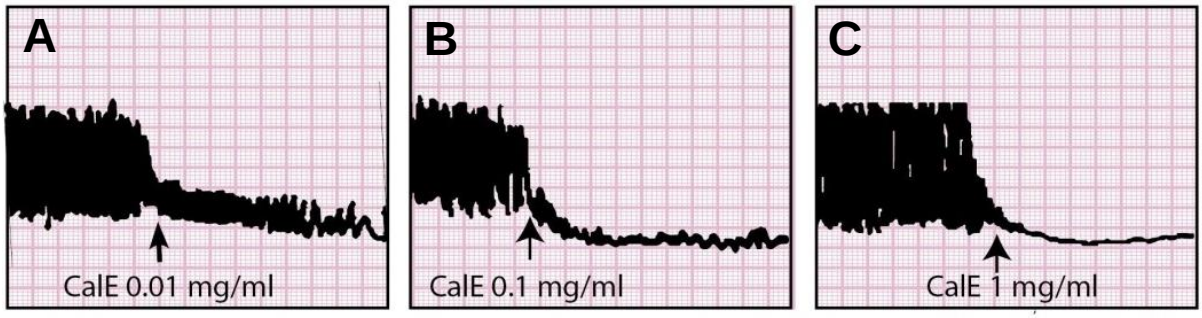

Fig. 4. Spasmolytic activity of hydroalcoholic extract of *Calendula arvensis* L (CalE) and possible role of muscarinic and nicotinic receptors. A-C) the effect of *Calendula arvensis* L extract (CalE), at different concentrations (0.01, 0.1 and 1 mg/mL) on the motility of isolated rabbit's duodenum. (Arrows refer to site of addition of the extract)

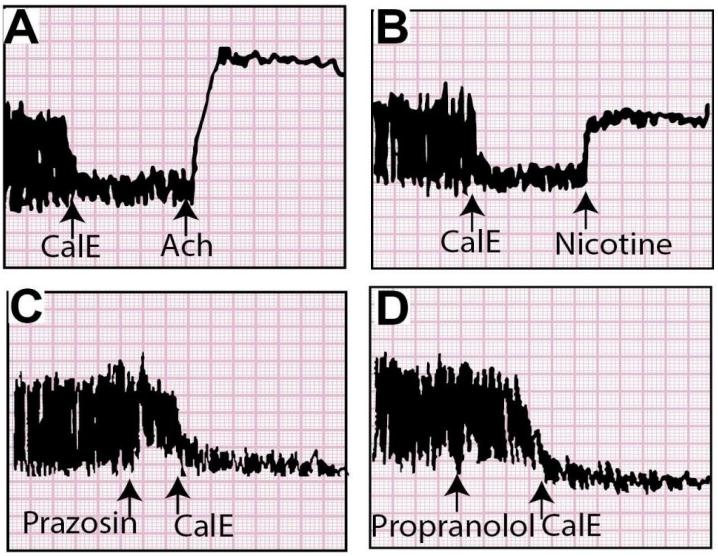

Fig. 5. Potential role of Adrenergic and cholinergic pathways and Calcium Chloride on spasmolytic activity of hydroalcoholic extract of *Calendula arvensis* L (CalE). **(A)** The effect of acetyl choline (Ach) at 5x10-7 M concentration after *Calendula arvensis* L extract (CalE) at 0.1 mg/mL on the motility of isolated rabbit's duodenum**. (B)** The effect of nicotine sulfate at a concentration of 2x10-5 M after CalE at 0.1 mg/mL bath on the motility of isolated rabbit's duodenum. **(C)** The effect of *Calendula* ethanolic extract (CalE) (0.1 mg/mL) after α adrenergic blocker Prazosin (4 x10-4 mg/mL bath) on the motility of isolated rabbit duodenum. **(D)** The effect of *Calendula arvensis* L extract (CalE) (0.1 mg/mL) after β blocker Propranolol (4 x 10-4 mg/mL bath) on the motility of isolated rabbit duodenum.

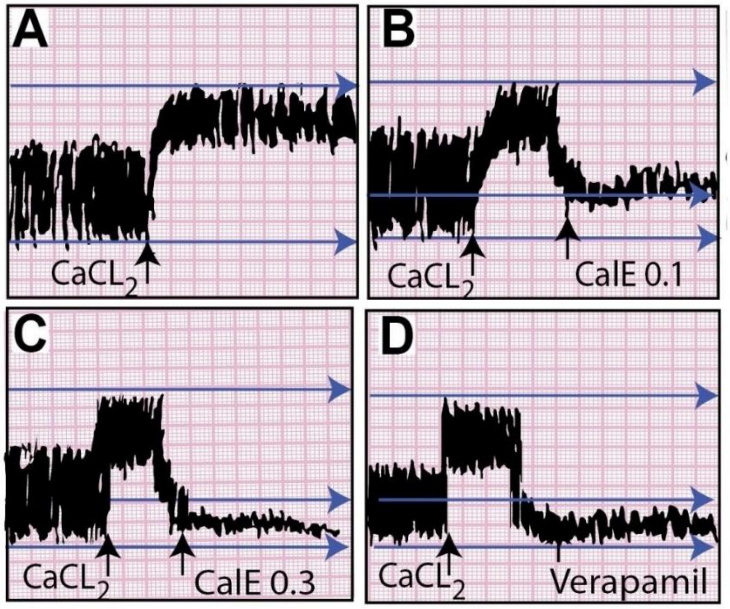

Fig. 6. Potential role of calcium channels on spasmolytic activity of hydroalcoholic extract of *Calendula arvensis* L (CalE). **(A)** the contractile response induced by CaCl2 in the absence of any treatment, CaCl2 at 5x10-3 M concentration. **(B-C)** The dose-dependent inhibitory effect of CalE at concentration of 0.1 and 0.3 mg/mL on the CaCl2-induced contraction. **(D)** the complete blockade of CaCl2-induced contraction with Verapamil, a known calcium channel blocker, serving as a positive control.

Discussion

Initially, this study evaluated Cal's LD50. Based on the mathematical calculation, Cal LD50 was determined to be 2450 mg/kg B.wt. Osweiler et al (Osweiler et al., 1985) reported that plant extract with LD_{50} below 10 mg/kg B.wt is highly toxic and others with LD_{50} above 50 mg/kg body weight are considered non-toxic. Based on Osweiler et al., (1985) LD_{50} values, this flower extract is evidently nontoxic and therapeutically safe.

Diarrhea is a gastrointestinal disorder associated with alteration of intestinal motility and fluid accumulation (decrease in absorption of fluid) within the intestinal tract. Diarrhea is manifested by frequent defecation of semisolid or liquid fecal material. Diarrhea could be fatal if it is associated with severe loss of fluid and electrolytes particularly sodium. From the therapeutic approach, spasmolytic and antisecretory agents are considered to be the mainstay agents used to decrease diarrhea-induced pathophysiologic-alterations.

Castor oil-induced diarrhea is a standard approach to investigating the antidiarrheal effect of a novel compound. Castor oil-induced diarrheal model is characterized by an imbalance between the secretory activity and absorptive processes in the small intestine by triggering inflammation and local irritation. Immediately after castor oil reaches the intestine, the active constituent of castor oil, "Ricinoleic acid", will be liberated by the action of intestinal lipases. After which, Ricinoleic acid will target and activate intestinal smooth-muscle-cells, through triggering "endothelial prostaglandin E3 receptor (EP3) receptors". As a result, it elicits the secretion of fluids and electrolytes, which serves as a secondary response to the stimulation of an active process involved in the secretion of anions, mediated by cyclic adenosine monophosphate (cAMP). Consequently, the utilization of castor oil to induce diarrhea in all models is deemed appropriate, as it closely mimics the pathophysiological alterations observed in both human and animal cases of diarrheal diseases, encompassing secretory and inflammatory forms.

In the present study, an investigation was conducted on the effects of CalE, derived from the flowers of CAL, on the frequency of mean fecal output induced by castor oil. The results revealed a significant reduction ($P < 0.01$) in fecal output frequency upon administration of the plant extract. Moreover, the inhibition of defecation exhibited a dose-dependent pattern, with the highest percentage of inhibition observed at a dose of 600 mg/kg of CalE. This observation suggests that a higher dosage of CalE is associated with a more effective antidiarrheal effect, comparable to that of atropine, a standard antidiarrheal drug.

Concerning the gastrointestinal motility test (charcoal meal intestinal transit test), CalE significantly ($P < 0.01$) inhibited the dynamic movement of the charcoal meal at all doses that have been used. CalE at doses of 200 mg/kg represents similar efficacy to atropine, a standard drug, while 400 and 600 mg/kg showed superior activity in comparison to atropine. This observation suggests that the administration of an even lower dosage may possess an adequate concentration of active constituents that are accountable for the

spasmolytic effect. On the other hand, at all dose ranges used (200, 400, 600 mg/kg), the CalE produced a significant ($P < 0.001$) inhibition of gastrointestinal content and fluid accumulation. This might indicate that the CalE possesses antisecretory activity, which reduces an excessive secretion executive by irritant effects of ricinoleic acid, an active metabolite of castor oil.

From a mechanistic aspect, the inhibitory effect of CalE appeared to be myogenic in nature, since the absence of atropine-like activity of CalE as ACh ($5x10^7$ M) induced its stimulatory effect in the presence of an effective inhibitory dose of CalE. Moreover, the absence of central cholinergic (ganglionic) antagonistic activity of CalE was proved by adding a stimulatory dose of nicotine sulfate $2x10^5$ M in the presence of an effective inhibitory dose of CalE which evoked its stimulatory effect indicating the absence of any ganglionic blocking activity of *Calendula arvensis* L.

In addition, the absence of α adrenoceptor activity of CalE was proved by inhibitory effect after blocking α receptors by Prazosin and the absence of β adrenoceptor activity of CalE was proved by inhibitory effect after blocking β receptors by Propranolol.

Conclusion

In conclusion, our study shows that the *Calendula arvensis* L. flower extract (CalE) has antidiarrheal properties in animal models. CalE effectively reduced the amount of wet feces and demonstrated significant antisecretory effects at all tested doses. We also evaluated the extract's safety through acute toxicity tests, which confirmed its safe use in traditional medicine. This research highlights the therapeutic potential of Calendula species as both an antidiarrheal and spasmolytic (muscle-relaxing) agent. For the first time, we explored the underlying mechanisms of these effects, providing scientific evidence to support the traditional use of this plant for treating diarrhea and muscle spasms. Our findings establish a scientific basis for using *Calendula arvensis* L. as a natural remedy for diarrhea and related intestinal issues. This study bridges the gap between folk medicine and scientific understanding, validating the plant's traditional applications with modern research methods.

Conflict of interest

All authors have none to declare.

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