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Original Research Article

HPLC Profile and Different Pathways Involved in *Achillea odorata*-Induced Gastric Emptying and Intestinal Transit Delay

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ABSTRACT

The *Achillea* genus is often used in traditional medicine for the treatment of digestive disorders. The current research sought to understand the mechanisms of action of the decocted extract of *Achillea odorata* L., with a focus on its possible effects on neurotransmitters that control gastrointestinal motility, as well as how it affected intestinal transit (IT) and gastric emptying (GE). Mice were given ADE treatments at 100, 200, or 400 mg.kg^{-1} doses, and an hour later they were given phenol red meal. To determine the effect of the extract on IT and GE, rats were given ADE (200 mg.kg^{-1}) in a different set of experiments while also receiving different pharmacological agents, such as atropine (3.45 mmol.kg^{-1}), L-Nitro-N-Arginine (L-NNA) (1.36 mmol.kg^{-1}), or indomethacin (5.58 mmol.kg^{-1}). At doses of 100, 200, or 400 mg.kg^{-1}, ADE showed a significant decrease in GE and IT; the corresponding values for GE were 45.62±2.69%, 42.92±4.91%, and 28.80±3.02%, respectively. and, similarly, 57.87±3.97%, 48.72±2.01%, and 42.81±3.96% for IT. These effects on GE delay and antimotility activity were mediated through the cholinergic, nitric oxide, and cyclooxygenase pathways induced by ADE. A chemical analysis of ADE using high-performance liquid chromatography coupled with a photodiode array detector (HPLC–DAD) revealed the presence of 12 phenolic acid compounds. The predominant phenolic compound identified in *A. odorata* was chlorogenic acid, with a concentration of 33.43±0.18 mg.g^{-1}. These results suggest that components of *A. odorata* L. may have potential applications in controlling gastrointestinal motility problems, such as diarrhoea.

Keywords: *Achillea odorata* L., decocted extract, chemical profile, gastric emptying, intestinal transit, mice

Introduction

Medicinal plants have been used historically to treat a variety of human ailments in traditional methods. As gastrointestinal disorders have the potential to have a major negative impact on health and even result in death all over the world, there is increasing interest in discovering novel compounds that can effectively treat these disorders. [1] Research from the previous year concentrated on treating and preventing digestive tract disorders with traditional medicine. [2]
The digestive system fulfils several vital roles that allow the body to get the nutrients it needs, such as vitamins, minerals, water, proteins, carbohydrates and electrolytes. Numerous neurotransmitters and mediators are involved in the complex process of controlling GE and IT. [3] Any disruption to those mechanisms leads to a variety of ailments, such as diarrhoea and constipation. Certain pathophysiological complications of the gastrointestinal tract are known to be influenced by dysfunction of gastrointestinal motility. [4]

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Both synthetic and natural medicines have been used to treat several metabolic and health problems brought on by irregularities in stomach motility and acid production. Over two millennia have passed since medicinal plants were first used, and in recent years, consumers' interest in herbal medicine products has increased because of their effectiveness and affordability. [5] Out of a total of 141 distinct medicinal plants, 34% are specifically employed for the treatment of gastrointestinal ailments. [6]
Within the Compositae family, the genus *Achillea,* which contains more than 120 species, is very important. There have been several reported results for *Achillea*, including anti-inflammatory,[7] anti-bacterial, [8] antihypertensive, [9] and antitumor. [10] There are a few reports of its effects, such as antispasmodic [11] choleretic [12] antiulcer effects on the gastrointestinal tract, and it is commonly used in conventional medicine to treat digestive issues. [11]
A. odorata L. *is* a medicinal plant, largely distributed throughout the Mediterranean region, [13] traditionally used to treat anti-inflammatory actions (rheumatism and allergic rhinitis) and digestive disorders, however, there is currently no scientific evidence to support its impact on gastrointestinal motility and gastric emptying.
The effect of *A. odorata* L on GE and IM is currently accessible. Therefore, the purpose of this study was to examine, the potential effects of a decocted extract of *A. odorata* L. in mice, as well as its mechanisms of action. The study aimed to assess whether it might interfere with the neurotransmitters that control gastrointestinal motility. Additionally, a chemical investigation was carried out to identify the primary active compounds of this species and their effects on gastrointestinal motility.

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Materials and Methods

Plant Collection and Identification

A. odorata L. was collected from Jijel, located in north-eastern Algeria in June 2020, It was identified and authenticated by a taxonomist (Pr. Amira S.) at the Laboratory of Phytotherapy Applied to Chronic Diseases where a voucher with the identification number 302 AO 16/06/20 Jij/SA was assigned. The plant was air-dried for approximately 10 days to ensure its proper preservation and pulverised using an electric grinder (RIRIHONG Brand Hi-speed Multifunctional Grinder, JAPAN).

Animals

Albino Swiss mice weighing 25-30 g were used in this study. Procured from the Pasteur Institute in Kouba, Algiers. Initially, they were housed in cages for a week in a typical lab setting under controlled environmental conditions, including temperature, and humidity, and were given free reign over their libido, food (ONAB, Algeria) and water for a week. Each animal was kept separately in cages with bottoms made of broad-mesh wire before the experiment began. The rats were provided with unrestricted access to water until 60 minutes before the trial commenced. However, they were subjected to a fast from a standard rodent diet for a duration of 18 to 20 hours. During this fasting period, the rats were not given any access to solid food.

The European Union's Guidelines for Animal Experimentation (2007/526/EC) were followed in the treatment of the animals. The Scientific Council of the Faculty of Natural Sciences and Life, University Setif-1 (Algeria), approved the animal experimentation portion of this study while upholding ethical considerations.

Methods

Preparation of decocted extract

The aqueous extract was made following the methodology outlined by mamache *et al.*[14] A 15 g of the powdered plant material was heated to a boil in 500 mL of water for ten (10) minutes, and then it was allowed to cool. The homogenate was then filtered using filter paper (Whatman size 2) and transferred into plates; it was dried at 38°C, [14] in an oven (MEMMERT UM 400 ref. P2209-1894, Germany). The extract obtained was stored at 4°C in dark, sealed bottle to prevent any molecular breakage due to light.

HPLC-DAD analysis

The phenolic compounds were analysed using the HPLC-DAD (Shimadzu 20 AT series cooperation, Kyoto, Japan) technique, which includes an Intertsil-ODS-3 reverse phase (C18) column.[15, 16] The rate of solvent flow was consistently maintained at 1.0 mL/min, while the amount of the sample injected was 20 µL. The mobile phases A and B were both composed of a solution containing 0.5% of acetic acid. The elution gradient used in this study consisted of several steps. Initially, the mobile phase composition was set at 0% B and gradually increased to 10% B within the time range of 0 to 0.01 minutes. Subsequently, the proportion of B was further increased from 10% to 20% throughout 0.01 to 5 minutes. This was followed by a gradual and constant increase in B from 20% to 30% over the time interval of 5 to 15 minutes. The gradient then continued with a stepwise increase in B from 30% to 50% (15 to 25 minutes), 50% to 65% (25 to 30 minutes), 65% to 75% (30 to 40 minutes), and 75% to 90% (40 to 50 minutes). Finally, the mobile phase composition was decreased from 90% to 10% B within the time range of 50 to 55 minutes.

The detection was performed using a photodiode array detector (PDA), and set to 280 nm. To characterize phenolic compounds, UV measurements and retention periods were compared to industry standards. The analysis was conducted thrice to ensure accuracy. To determine and measure the phenolic compounds, a calibration curve was established by the injection of reference chemicals at predetermined concentrations (0.0, 0.00782, 0.01563, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1.0 ppm). The findings were reported in terms of grams per gramme of dry mass.

Gastric emptying and small intestine transit in mice

Measurements of GE and small IT were conducted following the protocol outlined by Amira *et al.*[17] The test meal was composed of 1.5% carboxymethyl cellulose (CMC) and 0.1% phenol red, which was used as a non-absorbable visual marker. The test meal was given to the animals in a 0.3 mL volume. After twenty (20) minutes, the animals were subjected to euthanasia so that additional study and analysis could be done on them.

Subsequently, a laparotomy was performed to remove the stomach and small intestine, with the strangulation of the pylorus and cardia. The contents of the stomach were homogenised by using a 25 mL solution of 0.1 N NaOH. To induce protein precipitation, 8 mL of the liquid remaining after the homogenization process was merged with 1 mL of trichloroacetic acid solution (33%, p/v). Then, the resulting combination was then left to sit at ambient temperature for duration of one hour. The absorbance (Abs) was quantified at 560 nm (Shimazu uv-1800, Germany).

For each experiment, four animals were sacrificed immediately after consuming the test meal to serve as standards for 0% emptying. The rate of stomach emptying (GE) during the 20 minutes was calculated using the equation provided below (Eq. 1):

$$GE\ (\%) = (A_{Untreated} - A_{treated}/A_{Untreated}) \times 100 \quad (1)$$

Upon the removal of the stomach, the mesenteric attachments of the whole small intestine were mostly liberated, and its overall length was recorded. A drop of 0.1 N NaOH was used to accurately mark the exact position of the intestinal opening on the surface of the test meal. The ratio of the test meal's travel time to the entire small intestine length was used to calculate the rate of IT.

IT and GE of the meal were assessed in animals given one of the following drugs: atropine (3.4510-3 $mmol.kg^{-1}$), (L-NNA) (1.36 $mmol.kg^{-1}$) or indomethacin (5.5810-2 $mmoL.kg^{-1}$). The objective was to investigate whether these drugs could potentially disrupt the neural transmission that regulates gastrointestinal motility.

Statistical analysis

GraphPad Prism7.00 was used to conduct *in-vivo* statistical analysis. Results showed average standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to compare the values. Statistical significance was determined by using P-values that were less than 0.05.

Results and Discussion

Chemical investigation

A.odorata L. extract contained a high percentage of phenolic compounds which were identified and measured in $mg.g^{-1}$ of extract. Chlorogenic acid, ellagic acid, and luteolin are the most abundant compounds identified in the extract of *A. odorata*'s chemical constituents. The results are presented in Table 1 and Figure 1. The major phenolic compounds found in *A. odorata* L. were chlorogenic acid (33.43±0.18 $mg.g^{-1}$), ellagic acid (12.35±0.14 $mg.g^{-1}$), and luteolin (16.58±0.22 $mg.g^{-1}$).

Previous results, remarkably consistent with our findings for *Achillea millefolium*, revealed the presence of seven constituents (apigenin-7-*O*-glucoside, chlorogenic acid, vicenin-2, luteolin-7-*O*-glucoside, rutin, luteolin, and apigenin) using HPLC-DAD systems.[18] In addition to ellagic acid, luteolin, and other compounds, the HPLC results revealed that chlorogenic acid was a commonly occurring compound in the extract. Bobis *et al.*[19] quantified some phenolic compounds in *Achellia millefolium*, and the results showed that chlorogenic acid, rutin, and luteolin were the major compounds in the leaves of this species.

Additionally, research conducted on the aqueous extract of *Achillea santolinoides* L. revealed the presence of apigenin (apigenin-2''-*O*-pentosyl-8-C-glucoside and apigenin-*O*-glucuronide), luteolin-7-*O*-rutinoside, and dicaffeoylquinic acid (3,5 and 3,4-dicaffeoylquinic acid).

According to Birru et *al.*[20], constipation and diarrhoea are two opposing intestinal absorption, secretion, and motility diseases that result in unwarranted abdominal pain, discomfort, and a feeling of fullness, along with changes in bowel behaviours. Moreover, the stomach emptying process is disrupted and the changes to motility are two responses on a spectrum that have more severe consequences such as

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nausea and vomiting [21]. The present study thus focused on the dual effectiveness of *A. odorata* L. decocted extract on intestinal motility and GE.
The high bioactive content and diverse range of phenolic acids, flavonoids, coumarins, terpenes, lignans, and essential oils present in the *Achillea* genus have led to the attribution of biological activities such as antioxidant, antiulcerogenic, antibacterial, antispasmodic, immune suppressive, antitumor, and anti-diabetic effects.[22,23] In different parts of the world, different species of the genus are traditionally used for abdominal pain, flatulence, wound healing and preventing diarrhoea, acting as a diuretic, and treating wounds.[24] Chemical substance levels and concentrations have drawn attention because of their significant effects on the gastrointestinal tract.

Pathways of ADE-induced intestinal transit and gastric emptying delay
Figure 2 shows the results of various dosages of ADE on intestinal motility. Indeed, ADE is dose-dependent and significantly decreased the IT (45.62±2.69%, 42.92±4.91%, 28.80±3.02%) at 100, 200, and 400 mg.kg^{-1} respectively compared to the control group (62.31%±2.34; P≤0.0001).

Table 1: The phenolic composition of *A .odorata* L. decocted extract by HPLC-DAD (mg.g^{-1})^{a}.

Phenolic compounds	Retention time (min)	*A. odorata*
Protocatechuic acid	8.75	2.24 ± 0.02
Chlorogenic acid	12.35	33.43 ± 0.18
p-hydroxy benzoic acid	12.77	1.80 ± 0.06
6.7-Dihydroxy coumarin	14.10	0.59 ± 0.03
Coumarin	24.49	7.95 ± 0.08
Rutin	25.30	6.12 ± 0.05
Ellagic acid	26.11	12.35 ± 0.14
Rosmarinic acid	26.77	3.50 ± 0.06
Myricetin	27.35	0.87 ± 0.02
Luteolin	31.70	16.58 ± 0.22
Kaempferol	33.21	4.10 ± 0.08
Apigenin	33.77	3.95 ± 0.03

^{a}Values are expressed as means ± S.E.M. of three parallel measurements (p < 0.05). -: not detected

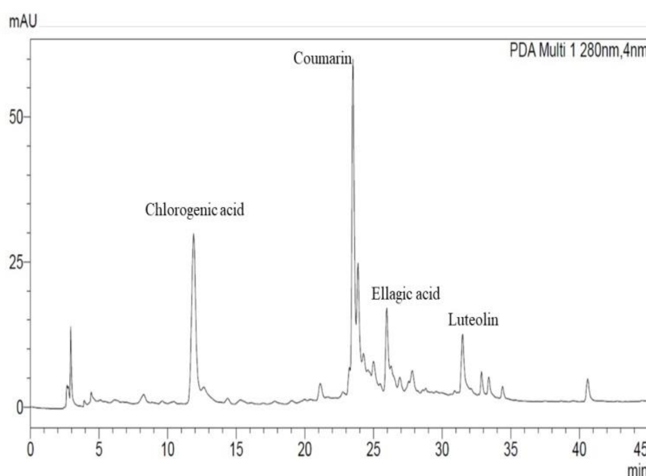

Figure 1: HPLC-DAD chromatogram of A. odorata L. decocted extract.

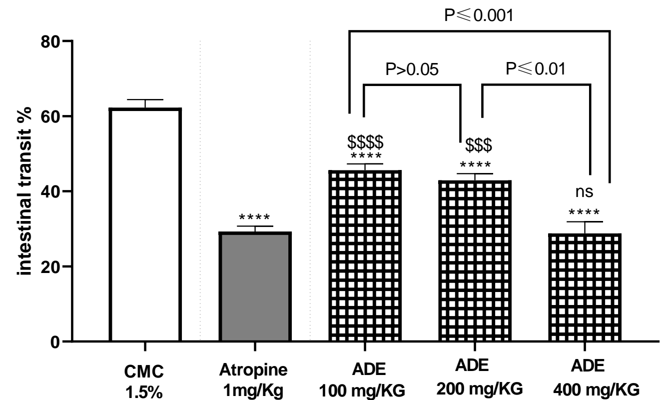

Figure: Effects of the ADE on intestinal transit in mice.
ADE; *A. odorata* L. decocted extract. Means ± SEM (n=9) is illustrated by bars. ****; P≤0.0001 vs vehicle (negative control). $$$; P≤0.001, $$$$; P≤0.0001 vs atropine (positive control). ns; not significant (P>0.05).

Furthermore, as predicted, antagonist effects were detected after the usage of standard; Atropine decreased IT assessment (29.29±3.11%). Compared to the atropine standard, only the dose of 400 mg.kg^{-1} displayed no significant difference (P>0.05).
The results reveal that the oral administration of *A. odorata* L. DE produced relaxing actions on the GE contents in the duodenum and resulted in a reduced effect on the GE process, as shown in Figure 3. Pre-treatment of mice with the sample at studied doses (100, 200, or 400 mg.kg^{-1}) reduced this action in a dose-dependent manner (80.11±3.99%, 57.87±3.97% and 48.72±2.01%), when compared to the control group (42.81±3.96%). When compared to atropine, a positive control (44.262.73%), ADE at 200 or 400 mg.kg^{-1} didn't differ significantly (P>0.05).
To ascertain the extract's mode of action on IT and GE, ADE (200 mg.kg^{-1}) was administered in a separate set of experiments while being accompanied by several pharmacological substances. Atropine (Atr) (3.45×10-3 mmol.kg^{-1}), L-NNA (1.36 mmoL.kg^{-1}) or indomethacin (Indo) (5.58×10-2 mmol.kg^{-1}) the administration led to a significant reduction (P≤0.0001) in IT to 29.29%, 35.99% and 44.27%. For Atr, L-NNA, and Indo, respectively (Figure 4), these pharmacological products reduced GE rates to 42.82%, 33.78%, and 43.58% (P ≤0.0001) (Figure 5).
The blockage of muscarinic receptors by Atr in the presence of ADE induced a significant (P≤0.0001) diminution (27.93±2.36%) on the IT compared to the vehicle (CMC 1, 5%). This IT observed with Atr remains lower (P≤0.0001) than that obtained with the extract alone while it had no considerable variation (P≤0.05) compared to animals exclusively receiving Atr.
Treatment of mice with L-NNA (NOS inhibitor) and ADE had significantly reduced IT (36.86±2.87%, P≤ 0.0001) when compared to CMC 1,5% and had no significant difference compared to the effect of L-NNA alone (P>0.05), similarly, the intra-peritoneal administration of indomethacin (inhibitor of prostaglandins production) with ADE reduce (P≤ 0.0001) the IT (41.62±2.13%) compared to the vehicle and remains unchanged when compared to the extract alone. The IT rate of ADE in the presence of Indo is comparable to that of Indo alone.
A significant diminution was observed using Atropine in the presence of the extract at the dose of 200 mg.kg^{-1} compared to the vehicle (P≤0.0001) and to the extract alone (P≤0.01). The GE in this case was 34.20%. Moreover, no significant difference (P>0.05) was recorded between Atr + ADE and Atr alone.
Esophageal administration of the ADE significantly reduced GE (24.8%; P<0.05) when NOS was inhibited. The ADE effects in the presence and absence of L-NNA differ significantly from one another (26.13%; P≤0.01). No discernible difference between ADE effects vehicle animals pretreated only with L-NNA was observed (35.05%; P>0.05). Moreover, the combination of extract with indomethacin remains non-significant compared to the group of indomethacin alone (34.08±1.13; P>0.05).

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Mechanisms that either suppress or enhance gastric motility action, pylorus, and intestine mediate GE and intestinal motility[24]. The results of this study showed that ADE pre-treated mice strongly, and in a dose-dependent manner altered the movement of the intestinal tract and emptying of the stomach in contrast to the controls. The fundus was investigated as a marker of fundus functionality during GE of a phenol red meal because of the increase of the intragastric and the gradient of peptic pressure generated by its tonic contractions.[25] Indeed, the size of the meal, the caloric density, the amount of water consumed, the composition of food, the administration of medications, the dimension of the particles, the state of medical care, and stress all have an impact on how quickly the stomach empties.[26]

While the extract's delaying effects on IT may be due to muscle contraction inhibition and/or expansion of the gut muscle's inhibitory factors, the extract's delaying effects on GE may be due to stomach musculature relaxation and/or pyloric sphincter constriction.[27]

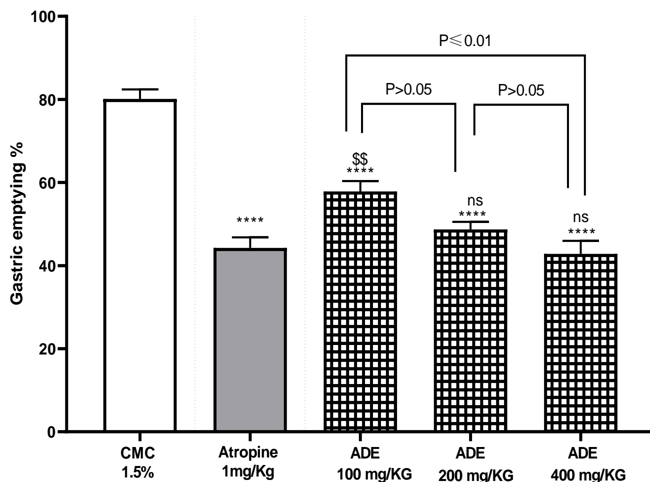

Figure 3: Effects of the ADE on gastric emptying in mice.
ADE; *A. odorata* L. decocted extract, Bars represent means ± SEM (n=9). ****; P≤0.0001 vs vehicle (negative control). \$\$; P≤0.01 vs atropine (positive control). ns; not significant (P>0.05)

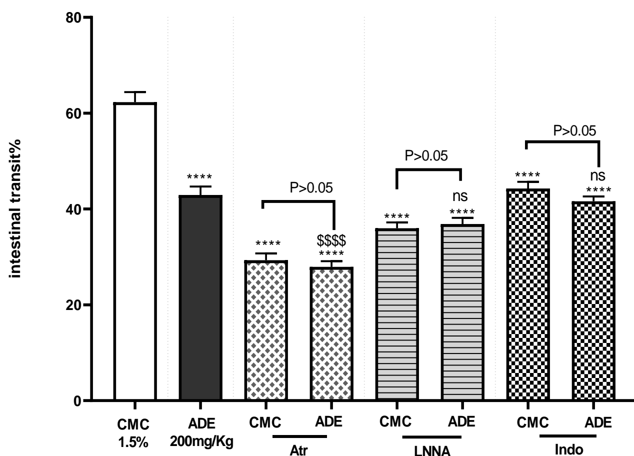

Figure 4: Effect of the ADE in the absence/presence of atropine, L-NNA or indomethacin on intestinal transit.
ADE; *A. odorata* L. decocted extract. Bars represent means ± SEM (n=9). ****; P≤0.0001 vs vehicle as negative control. \$\$\$\$; P≤0.0001 in comparison between both effects of ADE in absence and in presence of atropine, L-NNA or indomethacin. ns; no significant difference (P>0.05).

The study's findings concur with a 2011 study on the same genus conducted by Niazmand and Khoshnood [28], which revealed a blocking effect on the emptying of the stomach in both normal and vagal-promoted illnesses. The inhibiting impact might be brought about by an antagonistic effect on the flow of calcium from the intracellular storage in gastric smooth tissue or the acetylcholine-based flow of calcium. Additionally, Karamenderes *et al.* [29] demonstrated that *Achellia nobilis* extract had an antagonistic action on acetylcholine, which blocked the acetylcholine-induced smooth muscle contraction of rat duodenum. The effect of *A. santolinoides* L. aqueous extract on GE and IT was studied in mice.[30] It was found that the extract induced a significant delay in GE and in IT.

It has been established that polyphenols can relax the voluntary contractions of a variety of smooth muscles, including those found in blood vessels [31], bladder muscles [32], and uterine muscles. [33]. In the gastrointestinal tract, polyphenols have an inhibiting effect on the contraction of smooth muscle cells. Indeed, this strong inhibitory effect of ADE against the contraction of gastrointestinal muscles may be due to the presence of a high percentage of chlorogenic acid in the extract and this is in agreement with a study that was carried out by Posłuszny *et al.* [34] which confirms the antispasmodic effect of chlorogenic acid against contraction induced by acetylcholine. Furthermore, apigenin detected in the extract is an active inhibitor of the contractions brought on by LTD4. [35] According to previous research, the impact of this flavonoid is associated with calcium. The relaxation of gastric smooth muscles in mice has been seen to be influenced by the concentration of apigenin in the stomach, indicating that this effect is primarily a result of their capacity to block the entry of calcium via calcium channels with voltage regulation.[36] On the other hand, the delaying effect of GE is probably due to the blocking of muscarinic receptors by rosmarinic acid, which can inhibit these receptors in the mice's ileum.[37] Intraperitoneal administration of flavonoids found in our extract (apigenin, myricetin, and rutin), decreased IT by 28–69% in mice.[38] Polyphenols also affect IT, which has antidiarrheal effects. Atropine, a muscarinic receptor competitive antagonist of acetylcholine, can inhibit stomach emptying and small intestine motility.[39] According to Bahekar *et al.* [40], the role of the methods is probably to block M1 receptors on gastric parietal cells and aid in decreasing gastric secretions. Additionally, it inhibits M3 receptors on the stomach and intestine's visceral smooth muscles, causing these muscles to relax and lowering the amplitude and tone of these organs.[40, 41] The muscarinic receptor blockade is entirely responsible for the extract's inhibitory effect, which was nearly identical to atropine's control dose. Therefore, muscarinic receptors may play a role in how ADE prevents the emptying of the stomach and movement of the intestines.

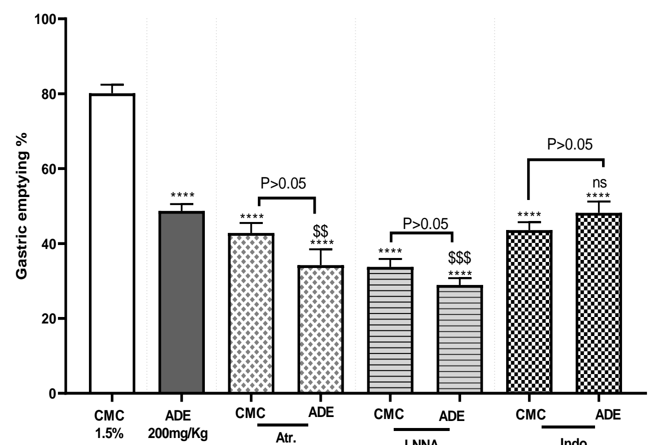

Figure 5: Effect of the ADE on gastric emptying in the presence or absence of atropine, L-NNA or indomethacin
ADE ; *A. odorata* L. decocted extract. Bars represent means ± SEM (n=9). ****; P≤0.0001 vs vehicle as negative control. \$\$; P≤0.01, \$\$\$; P≤0.001 in comparison between both effects of ADE in the absence and in the presence of atropine, L-NNA or indomethacin. ns; no significant difference (P>0.05).

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The NO synthase (NOS) produces NO from the amino acid L-arginine.[42] So far, at least two different types of NOS have been discovered. A constitutive NOS (cNOS) that responds to receptor stimulation by briefly releasing NO and is Ca^{2+}/calmodulin dependent. The inducible NOS (iNOS) is Ca^{2+}-independent and produces NO for extended periods after being expressed. Certain L-arginine analogues inhibit both enzymes, whereas glucocorticoids prevent the induction of iNOS.[43] NO is a neurotransmitter that is neither cholinergic nor adrenergic. It functions as a mediator of some substances' effects on gastrointestinal motilities. Intestinal motility and GE are delayed by L-NNA pre-treatment (inhibitor of cNOS).[44] These findings imply that constitutive and inducible NO synthase inhibitors have a mediating role in the ADE-induced inhibitions of the IT and GE, suggesting that this effect is dependent on the NO pathway. In the current study, similarly, the inhibitory effects of ADE on IT and GE were decreased with prior administration of L-NNA.

Cycle-oxygenase (COX) enzyme converts Arachidonic acid (AA) into endogenous prostaglandins (PGs). It is commonly known that PGs can cause the contraction of gastrointestinal smooth muscle and that their complex pharmacological actions can be used to modulate gastrointestinal motilities. Prostaglandin F2 (PGF2) accelerated the rate at which liquids are expelled from the human stomach, and the PGE series seems to relax the smooth circular muscle layer and contract the longitudinal smooth muscle layer. PGF series, in contrast, causes both small muscle layers to contract.[45] Both PGE2 and PGF2 stimulate IT and result in diarrhoea, but PGI2 does not.[46] As a COX inhibitor, indomethacin inhibits the production of PGs from AA and has antispasmodic properties. Pre-treatment of animals with indomethacin in this study attenuated both GE inhibitions and IT delaying caused by ADE. These findings suggest that endogenous PGs play as mediators of ADE effects on GE and IT, and they may also suggest a role for the cyclooxygenase pathway.

Conclusion

This study reveals that the phenolic compounds present in *Achellia odorata* L. potently reduce the gastric emptying and intestinal motility of mice through cholinergic, nitric oxide and cyclooxygenase pathways. These results suggest that *Achellia odorata* L. may have anti-diarrheal and/or spasmolytic properties. Further work is presently being conducted to identify the compounds responsible for these effects and confirm the possibility of involvement of other pathways, such as nerve transmission and adrenergic receptors, in the inhibition of GE and IT.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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