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Vanillic acid reverses castor oil-induced diarrhea in BALB/c mice through cholinergic and calcium channel blocking effects

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Abstract: **Background**: Diarrhea is one of the leading cause of death in developing countries. The available pharmacotherapy is linked with adverse effects. Vanillic acid is the major constituent of *Vanilla planifolia* and *Vanilla tahetensis* which are used in complementary medicines for treatment in gastrointestinal irritations and spasms. **Objectives**: This study aimed to investigate the antidiarrheal effect of vanillic acid in castor oil-induced diarrhea in BALB/c mice. **Methods**: The effects of vanillic acid on the diarrheogenic activity, gastrointestinal transit (GIT) and intestinal fluid contents in BALB/c mice model were evaluated. Additionally, the possible role of nitric oxide (NO), prostaglandin E_2 (PGE_2), opioid and cholinergic pathways were also investigated. *In-vivo* experiments were performed for ratifying the cholinergic and calcium channel blocking effects of vanillic acid. **Results**: Various doses of vanillic acid (1, 3, 5, 7 and 10 mg/kg) showed 12.20%, 23.80%, 48%, 61.80% and 79.20% protection against castor oil induced diarrhea in mice and induced significant volume reduction in enteropooling test; 7.44%, 37.23%, 48.93%, 63.82% and 70.21%. Through atropine-sensitive effect it reduced the gastrointestinal transit by 49.82%, besides inhibiting PGE_2-induced diarrhea by 39% at 10 mg/kg dose. In *in-vitro* studies it showed spasmolytic effects on jejunum contractions: spontaneous (EC_{50} value=0.21 µg/mL), high K^+ (EC_{50} value=4.31 µg/mL) and carbachol-induced (EC_{50} value=0.25 µg/mL); and on ileum contractions: high K^+ (EC_{50} value=2.34 µg/mL) and carbachol-induced (EC_{50} value=0.16 µg/mL). Calcium response curves produced a similar effects to verapamil. Vanillic acid was found safe in mice up to 2 g/kg body weight. **Conclusion**: It reduced intestinal transit stimulated by muscarinic agonist and intestinal secretion induced by PGE_2. *In-vitro* experimental results showed vanillic acid's spasmolytic action on jejunum and ileum tissues, likely through VDCC inhibition and muscarinic receptor antagonism.

Keywords: Antisecretory; BALB/c mice; Diarrhea; Gastrointestinal transit time; $_L$-NAME; $_L$-arginine; PGE_2; Vanillic acid

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INTRODUCTION

The increased feces fluidity, three or more than three bowl movements per day (Dos-Santos *et al*., 2019) with increased gastrointestinal motility and secretion are the characteristics of diarrhea (Deane *et al*., 2019). Diarrhea has myriad causes including contaminated food and water, endocrine diseases, viral, bacterial and protozoal infections (Florez *et al*., 2020). According to duration, it is categorized into acute (1-13 days), persistent (≥ 14 days) and chronic diarrhea (> 30 days) (España *et al*., 2023). According to the physiological mechanism diarrhea is classified into secretory, osmotic, inflammatory and motor diarrhea (Kelly *et al*., 2018). Diarrhea is a preventable disease even then around 1.6 billion cases are reported worldwide every year. Death rates from diarrhea in Pakistan and India are 65.78 and 64.5 per 100,000 deaths, respectively (Ali *et al*., 2022). Diet, oral rehydration therapy, antidiarrheal agents and antibiotic therapies are consideration for the treating diarrhea (Sokic-Milutinovic *et al*., 2022).

The relationship between anti-inflammatory, antioxidant and antidiarrheal effects is interrelated and can work together to alleviate diarrhea. Pro-inflammatory mediators, such as cytokines, can irritate the intestinal lining and cause diarrhea by increasing fluid secretion, decreasing absorption in the gastrointestinal tract, and impairing motility in the intestine. Anti-inflammatory substances can help to minimize this inflammation and alleviate diarrhea. (Pérez *et al*., 2011). Oxidative stress can lead to inflammation and damage the cells lining of the intestinal tract, compromising the integrity of the intestinal barrier increasing its permeability (Vona *et al*., 2021). Antioxidants can play role in alleviating diarrhea by reducing oxidative stress, anti-inflammatory effect, preserving intestinal barrier function, supporting gut micro biota balance, improving immune function, modulating gut motility and reducing gastrointestinal discomfort (Tian and Piao, 2019). Literature review reveals many phenolic compounds comprising anti-inflammatory and antioxidant effects with antidiarrheal effects such as gallic acid, zingerone, ellagic and curcumin (Parham *et al*., 2020).

Vanillic acid is a phenolic compound and derivative of benzoic acid (Taqvi *et al*., 2021). Research has demonstrated its pharmacological potential as an anti-inflammatory, antioxidant, hepatoprotective, immunostimulant, cardioprotective and neuroprotective (Ingole *et al*., 2021). Vanillic acid's antiinflammatory effect is arbitrated via multiple mechanisms, including

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inhibition of NF-kB pathway, suppression of pro-inflammatory mediators such as cytokines (Haq *et al*., 2021). Vanillic acid has an antioxidant effect because it can scavenge free radicals, upregulate antioxidant enzymes and inhibit the activity of enzymes that cause inflammation and oxidative stress (Calixto *et al*., 2015; Taqvi *et al*., 2021). Vanillic acid is the major constituent found in extract of vanilla beans of *Vanilla tahitensis* and *Vanilla planifolia* (Ignat *et al*., 2011) having medicinal value in gastrointestinal spasms and irritations (Singletary, 2020). The purpose of the said study remained to explore antidiarrheal, antisecretory and antimotility activity of vanillic acid.

MATERIALS AND METHODS

Chemical

Chemicals were attained from the specified sources: charcoal meal, L-NAME, verapamil hydrochloride, loperamide HCl, L-arginine, prostaglandin E_2, acetylcholine chloride, atropine sulphate, castor oil (Karachi Chemical Industries, Karachi, Pakistan), verapamil hydrochloride and vanillic acid (Sigma Aldrich Chemicals Company, St. Louis, MO, USA).

Animals

The rules of institution of Laboratory Animal Resources, Commission of Life Sciences, NRC (National Research Council, 2011) and COMSATS University Islamabad, Abbottabad Campus were followed to perform experiments. BALB/c mice with weights of 20-25 g were utilized for this study.

Effect of vanillic acid on diarrhea induced by castor oil in mice

The respective study was performed by the methodology described previously (Umer *et al*., 2013, Bashir *et al*., 2023). After 18 hours fasting BALB/c mice were allocated into 8 groups comprising 5 mice in each group, housed in steel cages (bottom covered with filter paper). First group received 10 mL/kg vehicle, second group received 10 mL/kg of castor oil, 3th to 7th group were administered with the doses of vanillic acid (1, 3, 5, 7 and 10 mg/kg) and eighth group received 10 mg/kg of loperamide. Each group was administered with castor oil orally one hour after treatment and monitored for defecation. The mice were observed, after 4 hours, for the number of diarrheal spots and non-diarrheal spots. Percentage protection was calculated for each group by using a formula,

$$\%\ \textbf{Protection} = \frac{\textbf{Mean number of dry feces}}{\textbf{Mean defecation}} \times \mathbf{100}$$

Effect of vanillic acid on gastrointestinal fluid accumulation induced by castor oil in mice

The aforementioned study was investigated by pursuing the previously described method (Robert *et al*., 1976; Saqib *et al*., 2021). BALB/c mice were kept under fasting overnight. They were divided into eight groups containing 5 mice in each group and housed in steel cages and treated with following doses orally. The dose of 10 mL/kg of vehicle was administered to the first group, second group received 10 mL/kg of castor oil, third to seventh groups were administered with the doses of vanillic acid (1, 3, 5, 7 and 10 mg/kg) and eighth group received 10 mg/kg of loperamide. One hour later castor oil was administered to all groups. Cervical dislocation method was used to sacrifice mice thirty minutes later. Pyloric sphincter to ileocecal junction of small intestine of mice were cut off and carefully weighed. The results were calculated using the following formula.

$$\textbf{\textit{P}i/\textit{P}m} \times \mathbf{1000}$$

In the above formula, *P*i is the intestine weight and *P*m is the weight of the mice.

Contents in the intestine were collected in graduated syringe, measuring its volume and then calculated % reduction in volume of contents of intestine by the following formula,

$$\%\ \textbf{Reduction in volume of intestinal content} = \frac{\mathbf{A} - \mathbf{B}}{\mathbf{A}} \times \mathbf{100}$$

In the above formula, A is intestinal content volume triggered by castor oil, B is volume of contents of intestine after loperamide or vanillic acid treatment.

Percent reduction was calculated after carefully weighing the weight of contents of intestine using the following formula,

$$\%\ \textbf{Reduction in weight of intestinal content} = \frac{\mathbf{A} - \mathbf{B}}{\mathbf{A}} \times \mathbf{100}$$

In the above formula, A is intestinal content weight induced by castor oil, B is weight of intestinal content when treated with loperamide or vanillic acid.

Vanillic acid effect on L-arginine-induced intestinal fluid accumulation in mice

This study was investigated by pursuing the previously described method (Izzo *et al*., 1994, Elmongy *et al*., 2022). BALB/c mice were caged in steel cages after dividing them into eight groups containing 5 mice in each group after an overnight fast. First group received vehicle, second group was administered with 10 mg/kg vanillic acid and third group was administered with L-NAME. Secretions were induced by L-arginine (600 mg/kg, *p.o*.) from group 1 to 3 after an hour. After thirty minutes, pyloric sphincter to ileocecal junction of mice were removed via cervical dislocation, and carefully weighed, expressing the result using the formula,

$$\%\ \textbf{Reduction in weight of intestinal content} = \frac{\mathbf{A} - \mathbf{B}}{\mathbf{A}} \times \mathbf{100}$$

Vanillic acid effect on accumulation of gastrointestinal fluid induced by PGE_2 in mice

Vanillic acid effect on PGE_2 induced accumulation in small intestine was investigated, pursuing previously described method (Izzo *et al*., 1994, Beserra *et al*., 2016). BALB/c mice (n = 5) were kept on fasting for 6 hours. Vehicle was

given to group 1, group 2 received vanillic acid and loperamide was given to third group. After waiting for one hour, PGE_2 (100 µ/kg, *i.p.*) was administered in all mice from group 1 to 3, that induced intestinal fluid secretion. To measure small intestine (pyloric and ileocecal junction), all animals were killed 30 minutes after PGE_2 was administered. After the intestinal contents were evacuated, empty and full intestine difference was calculated.

Effect of vanillic acid on charcoal meal gastrointestinal transit time in mice

The *in-vivo* gastrointestinal transit time activity was explored, following previously described methodology (Aye-Than *et al*., 1989, Elmongy *et al*., 2022). Mice were fasted for twelve hours and after that they were divided into five groups, each group containing 5 mice, where following doses were administered. Mice in group 1 received vehicle, mice in group 2-6 received vanillic acid (1, 3, 5, 7 and 10 mg/kg) and loperamide was administered to 8th group. As a marker diet charcoal meal was given orally to each mouse an hour later. The mice were sacrificed an hour after being fed the charcoal meal, and their abdomens were cut open. The measurements were used to calculate the transport % age of charcoal using following formula,

$$\%\ \text{Distance travelled by charcoal meal} = \frac{\%\ \text{Distance traversed by the charcoal meal} \times 100}{\text{Small intestine length}}$$

Effect of vanillic acid on transit in intestinal tract via opioid system in mice

In-vivo opioid gastrointestinal transit time activity was conducted using previously described method (Dos-Santos *et al*., 2019). BALB/c mice (20-25g) were divided into 6 groups following an overnight fast (n=5). Castor oil (10 mL/kg) was administered to all groups. In groups without antagonist (naloxone), vehicle was administered to 1st groups, loperamide to group 2 and vanillic acid (10 mg/kg) to group 3 after thirty minutes. Groups with antagonist received naloxone (2 mg/kg, *s.c.*) after thirty minutes of castor oil induction. After 30 minutes, vehicle was administered to group 4 and group 5 and 6 received loperamide and vanillic acid and loperamide. Charcoal meal was administered (0.25 ml/mouse) after 1 hour to all groups. Mice were sacrificed after 30 minutes and the abdomen region was cut open. Percentage inhibition was measured.

$$\%\ \text{Inhibition} = \frac{N - n}{N} \times 100$$

In the aforementioned formula, 'N' and 'n' is charcoal meal distance in untreated and treated mice.

Vanillic acid effect on transit in intestinal tract via cholinergic system in mice

Cholinergic gastrointestinal transit time activity was explored by the method designed formerly (Dos Santos *et al*., 2019). Mice weighing 20 to 25 g were divided into five groups (n=5) after overnight fasting. Vehicle was given to first group, second group remained untreated, group 3 received vanillic acid, and fourth group was treated with atropine. After 30 minutes, acetylcholine (5 mg/kg, *i.p.*) from group 2-4 was administered. One hour later charcoal meal (mL/mice) was administered orally. The mice were sacrificed after 20 minutes and abdomen region will be cut open. Then percentage inhibition was calculated.

$$\%\ \text{Inhibition} = \frac{N - n}{N} \times 100$$

Acute toxicity test

This test was done as described earlier (Lorke, 1983). Each group was allocated with 5 mice. Increasing doses of vanillic acid (10, 50, 100 and 500 mg/kg) was given orally to mice. One group was taken as a control group to which normal saline was administered. These mice were observed for 24-hours for mortality.

In-vitro studies

Effects of vanillic acid on jejunum and ileum tissues of mice

The aforementioned activity was conducted via same methodology that was used previously on mouse jejunum and ileum tissue (Gilani *et al*., 2005). With unrestricted access to water, experimental mice were maintained on an overnight fast. Mice were sacrificed by severing their cervical vertebrae and opening their abdomens. Isolating segments of the ileum and jejunum, 2-3 cm in length, cleared the mesentery. A 10 mL tissue bath kept at 37 °C was used to mount the tissue preparations, and carbogen (5% carbon dioxide in oxygen) was used to aerate the solution.

1g preload was specified for jejunum tissue and 0.5-1 g preload was specified for tissues of ileum. Following a 30-minute equilibration interval, the tissues were considered stable based on the consistency of the aforementioned reactions. Upto 0.3 µM Ach sub-submaximal dose, control response was attained. In tissues that were at equilibrium, vanillic acid effect was confirmed by contractions brought on by 80 mM of high K^+ and carbachol.

Determination of Ca^{+2} channel blockade action of vanillic acid on mice jejunum

To determine the vanillic acid's blockade activity of calcium channel-blockade activity, jejunum of mice was used because of its rapid responsiveness. For stabilization of these tissues Tyrode's solution (normal) was used. Substitute this solution with Ca^{+2} free solution of Tyrode which contains 0.1 mM ethylenediaminetetra-acetic acid to make tissue bath free of calcium. Later, Tyrode's solution, which is rich in potassium and free of calcium, was used in its place for 30 minutes. Replicable cumulative $CaCl_2$ concentration response curves were obtained. To assess calcium channel blocking properties, the jejunum tissue was pretreated with different concentrations of vanillic acid (Mapesa *et al*., 2021).

Statistical analysis

In this research, results are articulated by way of mean and standard error means (SEM). For statistical execution, *p*-value (< 0.05) and one-way ANOVA followed by Dunnett's test and Tukey's test being deemed significant (Graph-Pad prism version 8, San Diego, Cao, USA).

RESULTS

Effect of vanillic acid on diarrhea induced by castor oil in mice

Vanillic acid significantly ($p < 0.05$) reduced diarrhea in a dose-dependent manner compared to the untreated group. Its protective effect was comparable to that of the standard drug, loperamide (Table 1).

Effect of vanillic acid on gastrointestinal fluid accumulation induced by castor oil in mice

Vanillic acid exhibited a dose-dependent antisecretory effect, significantly reducing castor oil-induced intestinal fluid accumulation ($p < 0.05$). Loperamide (10 mg/kg) also markedly decreased fluid accumulation. A corresponding dose-dependent reduction in the volume of intestinal contents was observed with vanillic acid ($p < 0.05$), while loperamide produced a greater inhibitory effect (Table 2 and 3).

Vanillic acid effect on L-arginine-induced intestinal fluid accumulation in mice

Vanillic acid reduced accumulation in intestinal fluid at 10 mg/kg by 2 % with L-arginine while L-NAME reduced it by 30% (Table 4).

Vanillic acid effect on accumulation of gastrointestinal fluid induced by PGE2 in mice

Vanillic acid reduced accumulation in intestinal fluid via PGE_2 by 29% while with loperamide it was 39% (Table 4).

Effect of vanillic acid on charcoal meal gastrointestinal transit time in mice

Vanillic acid produced a dose-dependent spasmolytic effect, significantly reducing the intestinal transit of the charcoal meal compared to the control group ($p < 0.001$). The reduction in gut motility was comparable to that produced by the standard drug loperamide (5 mg/kg), although loperamide demonstrated a slightly greater inhibitory effect ($p < 0.001$ vs. control) (Fig. 1).

Effect of vanillic acid on transit in intestinal tract via opioid system in mice

Vanillic acid significantly decreased intestinal transit compared to the control group ($p < 0.05$). Pretreatment with naloxone (2 mg/kg) did not significantly reverse the effect of vanillic acid, indicating no major involvement of the opioid pathway (Fig. 2).

Effect of vanillic acid on transit in intestine mediated by cholinergic system in mice

Vehicle and acetylcholine-treated mice showed significantly higher intestinal transit compared to vanillic acid and atropine-treated groups (*$p < 0.05$) (Fig. 3).

Acute toxicity test

In BALB/c mice, vanillic acid was safe up to 2 g/kg; no mortality was seen, and the mice's overall behavior and breathing were both regular.

In-vitro studies

Mice jejunum

Vanillic acid caused inhibition of spontaneous [EC_{50} = 0.210mg/mL (0.13-0.30)], high K^+ [EC_{50} =.4.314 mg/mL (3.22-5.32)] and carbachol (CCh) induced contractions [EC_{50} = 0.25 mg/mL (0.17-0.38)] in isolated mice jejunum tissue preparation (Fig. 4, 5, 7A-7H). Verapamil caused inhibition of spontaneous [EC_{50} = 0.09 mg/mL (0.06-0.16)] and high K^+ induced contractions [EC_{50} = 0.21 mg/mL (0.12-0.36)].

Mice ileum

Vanillic acid resulted in the inhibition of high K^+ [EC_{50} = 3.08 mg/mL (2.34-4.55)] and carbachol (CCh) induced contractions [EC_{50} = 0.1602 mg/mL (0.11-0.223)] in isolated mice ileum tissue preparation (Fig. 6, 7G-7I). The high K^+ induced contraction was inhibited by verapamil [EC_{50} = 0.22 mg/mL (0.13-0.39)]. Calcium channel blockade effect of vanillic acid was confirmed when pretreatment of the tissue with 0.1, 0.3, 1 µM/mL dose of vanillic acid caused a rightward shift in Ca^{++} concentration response curves (CRCs). Verapamil also shifted the Ca^{++} CRCs to the right.

DISCUSSION

To validate the antidiarrheal effects of vanillic acid as it is expected to alleviate secretory diarrhea due to its anti-inflammatory and antioxidant, this study was conducted. The antidiarrheal effect of vanillic acid was first studied in a diarrheal model induced by castor oil, which is considered one of the best models for studying secretory diarrhea due to its well characterized mechanism involving prostaglandin-mediated chloride secretion, its rapid and reproducible onset of symptoms, and its ability to mimic the electrolyte and water secretion patterns seen in human secretory diarrhea (Sarma *et al*., 2024). This model provides a reliable platform for testing the efficacy of anti-diarrheal agents (Oghenesuvwe *et al*., 2018).

The results obtained showed that all doses provided significant percentage protection against the castor oil induced diarrhea which indicated there inhibitory effect on secretion or motility or both. Pancreatic lipases metabolizes castor oil into ricinoleic acid and glycerol, ricinoleic acid induces enteropooling which amends

intestinal electrolyte and water transport causing diarrhea, hypersecretions and intestinal spasms particularly in distal and transverse colon. In addition, prostaglandins and platelet activating factors are also released by ricinoleic acid in the small intestine (Chauke *et al*., 2019; Dong *et al*., 2021). By promoting intestinal fluid and electrolyte secretion and preventing sodium absorption, prostaglandins induce diarrhea (Keely and Barrett, 2022). Prostaglandins also promotes vasodilation, mucus secretion and smooth muscle contraction resulting diarrhea (Gao, *et al*., 2022). Furthermore, ricinoleic acid increases nitric oxide production and adenylyl cyclase activation, which raises the concentration of cAMP and stimulates peristaltic movement in the colon (Gupta *et al*., 2024). It causes the intestinal lumen to fill with an accumulation of water and electrolytes by changing the membrane permeability and reducing Na^+K^+ATPase pump's activity (Dos-Santos *et al*., 2019). Thus vanillic acid was further explored on the two fundamental mechanisms that is intestinal secretion and motility.

Vanillic acid's antisecretory effect induced by PGE_2 was performed. Intestinal fluid production was considerably decreased by vanillic acid at every tested dose. Increased reabsorption of water and electrolytes, possibly through some sort of antisecretory mechanism, could account for this effect (Keely *et al*., 2022). By interacting with the EP_3 receptors present in contractile muscles, PGE_2 increases intestinal motility and promotes contractions (Kushwaha *et al*., 2024). PGE_2 also stimulates the chloride ions secretions via CFTR channel, increasing water and sodium into lumen of intestine and contributing to the watery stools associated with secretory diarrhea (Kinuthia *et al*., 2016). PGE_2-induced enteropooling analysis showed that vanillic acid led to a significant decrease in fluid production. Using activated charcoal, gastrointestinal transit time was measured to assess the impact of vanillic acid on intestinal motility. Comparing the results to loperamide, the conventional medication, indicated that vanillic acid substantially slowed gastrointestinal transit, suggesting a decrease in intestinal motility. Loperamide is an antidiarrheal medication that reduces smooth muscle contractions by binding to μ-opioid receptors (Sobczak *et al*., 2014).

To find out if the effects of vanillic acid are associated to opioid system, the transit in intestine regulated by the opioid receptor antagonist naloxone was evaluated. The impact of loperamide was then found to be greatly reduced by naloxone but it was unable to counteract the impact that vanillic acid had, indicating that its antidiarrheal action is independent of the opioid mechanism. This is advantageous since it eliminates any potential negative effects associated with opioid agonist action. The possibility that vanillic acid might function through cholinergic pathways was then investigated. When this neurotransmitter is inhibited, intestinal transit can be slowed. Acetylcholine (ACh), generated by the parasympathetic nervous system is involved in bowel movement's regulation. It does this by increasing the input of calcium and contracting smooth muscle (Wehrwein *et al*., 2016). Thus, cholinergic modulated transit was used to study vanillic acid potential anticholinergic effect. In order to cause intestinal contractions, acetylcholine, a cholinergic agonist, activates the numerous M_3 receptors found throughout the gastrointestinal system. The stimulatory effects of acetylcholine were significantly reduced by vanillic acid (*$p< 0.05$), which led to a decrease in intestinal transit (39.60 ± 1.67 %). This evidence demonstrates that vanillic acid directly competed with atropine, a cholinergic antagonist that decreases motility and prolongs intestinal transit time, for the M_3 receptors in the gastrointestinal tract (Teixeira Neto *et al*., 2012).

Based on *in-vivo* effects regarding vanillic acid, further *in-vitro* experiments were performed to see the possible spasmolytic effects on intestinal jejunum and ileum tissues in mice. Spontaneous effects of vanillic acid exhibited spasmolytic effect in concentration dependent manner in the absence as well as presence of spasmogen when tested against isolated jejunum tissue. The concentration-dependent suppression of contractions induced by high K^+ (80 mM) by vanillic acid and the similarity of the relaxation to verapamil imply that the mechanism may be the inhibition of calcium transport through VDCCs (Sagar *et al*., 2021).

Moreover, vanillic acid like verapamil caused a shift to the right in Ca^{++} CRCs (Elmongy *et al*., 2022), suggesting that VDCCs mediate the spasmolytic effect. Since carbachol is known to have an anticholinergic effect, when mice's jejunum and ileum tissue preparations were tested against contractions generated by carbachol, vanillic acid demonstrated spasmolytic properties, indicating anticholinergic effects (Saqib *et al*., 2021). According to the experimental findings, the antagonism between VDCCs and muscarinic receptors is likely responsible for vanillic acid's spasmolytic effect. Vanillic acid was shown to be safe up to 2 g/kg body weight after passing acute toxicity studies.

CONCLUSION

This study demonstrates the antidiarrheal potential of vanillic acid through both antisecretory and anti-motility actions. Unlike opioid based drugs, its effects appear to involve anticholinergic activity and VDCC inhibition, supporting reduced intestinal transit and fluid secretion. These findings suggest vanillic acid may serve as a safe and effective therapeutic option for managing diarrhea. Further research is warranted to clarify its underlying molecular mechanisms.

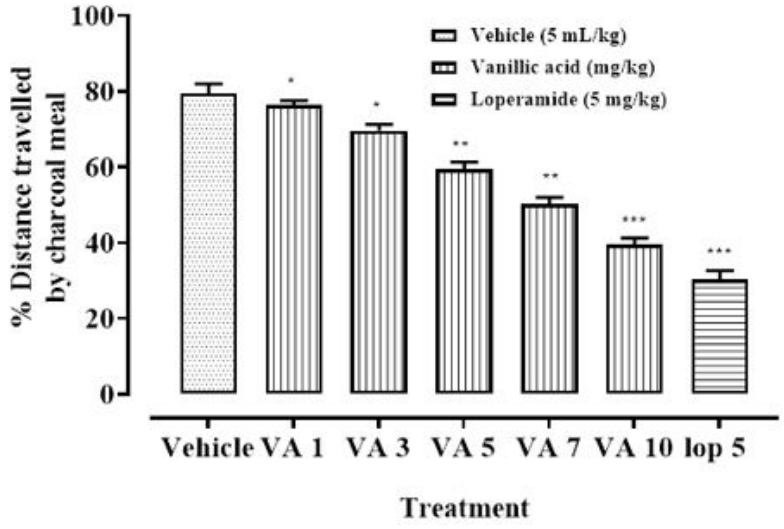

Fig. 1: Bar chart showing the effect of vanillic acid on charcoal meal gastrointestinal transit time in mice. Mean ± S.D (n=5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control (saline), One way ANOVA followed by Dunnett's-test.

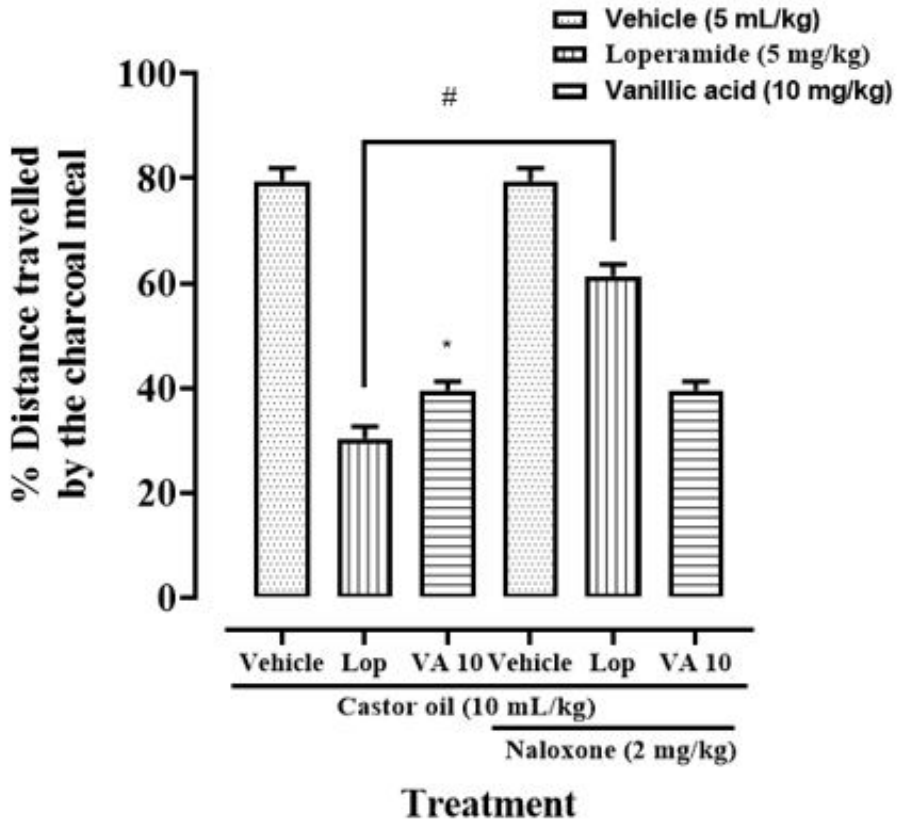

Fig. 2: Bar chart showing the effect of vanillic acid on the intestinal transit mediated by the opioid system in mice. Mean ± S.D (n=5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. One way ANOVA followed by Tukey's-test.

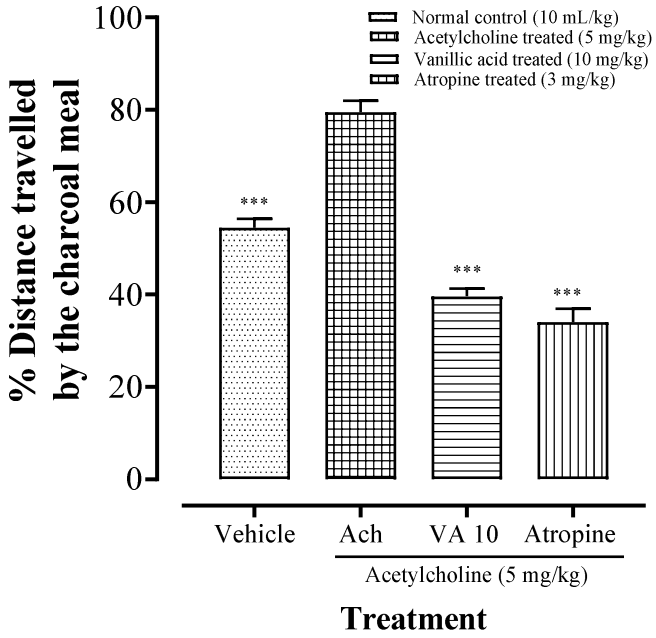

Fig. 3: Bar chart showing the effect of vanillic acid (VA) on the intestinal transit mediated by the cholinergic system in mice. Mean ± S.D (n=5).$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when comparing the groups; vehicle vs. vehicle + acetylcholine, vanillic acid + acetycholine, atropine + acetylcholine, One way ANOVA followed by Tukey's-test.

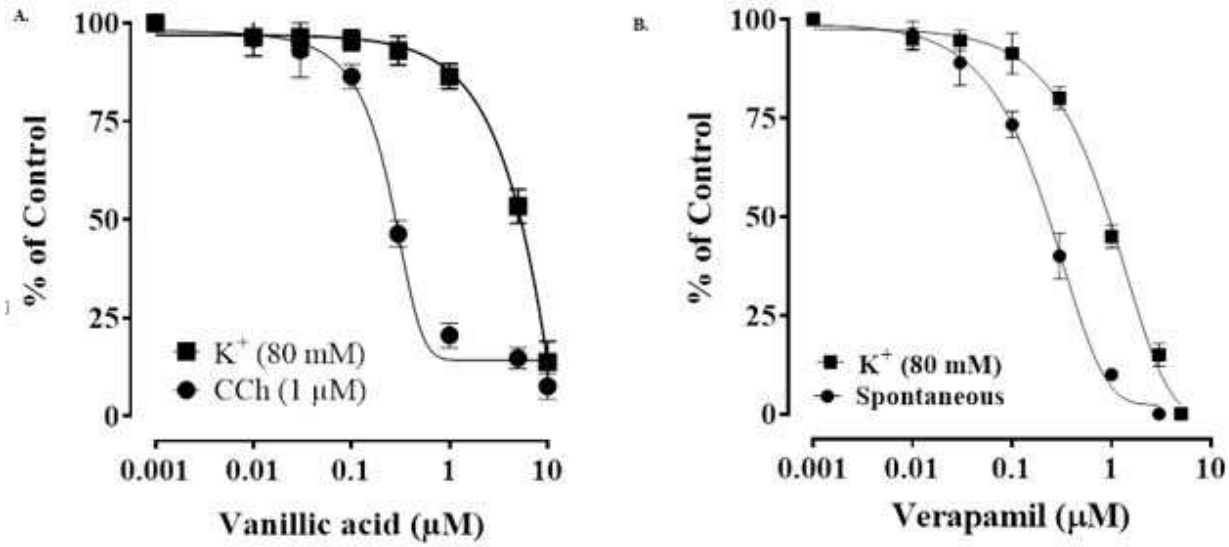

Fig. 4: Concentration response curves of (A) Vanillic acid against K+ (80mM) and carbachol (CCh)-induced contractions, (B) Verapamil against spontaneous and K^+ (80mM)-induced contraction in isolated mice jejunum preparations. Mean ± SEM (n=5).

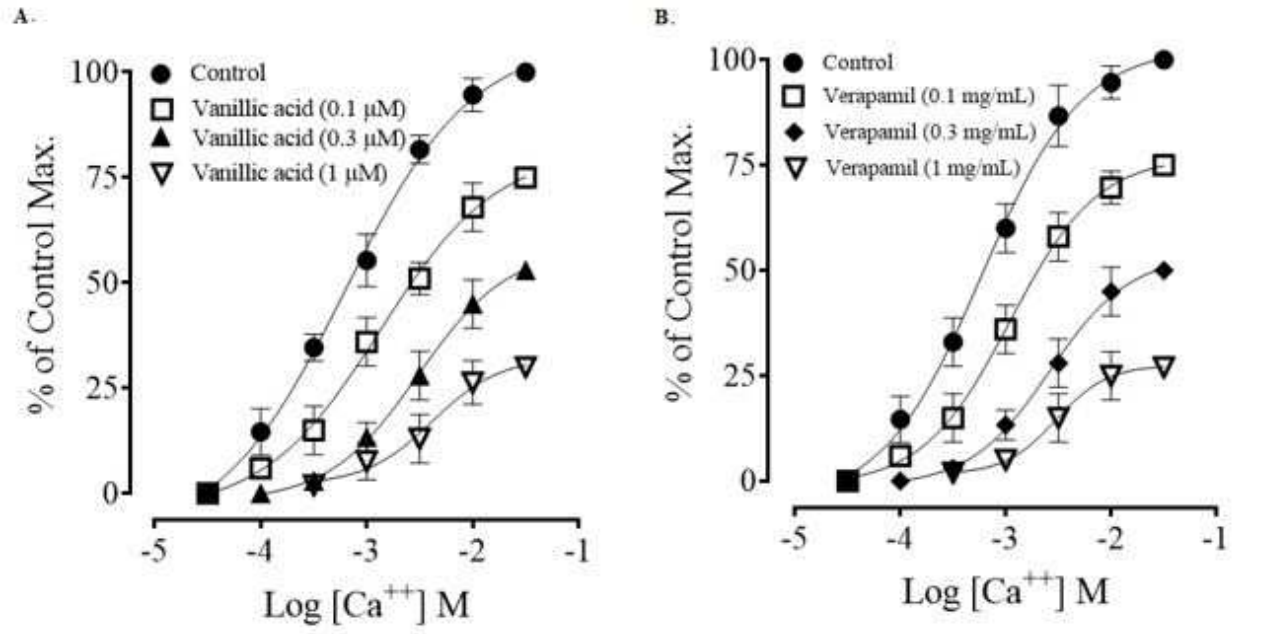

Fig. 5: Effect of different concentration of (A) Vanillic acid (B) Verapamil on Ca^{++} concentration-response curves in isolated mice jejunum preparations. Mean ± SEM (n=5).

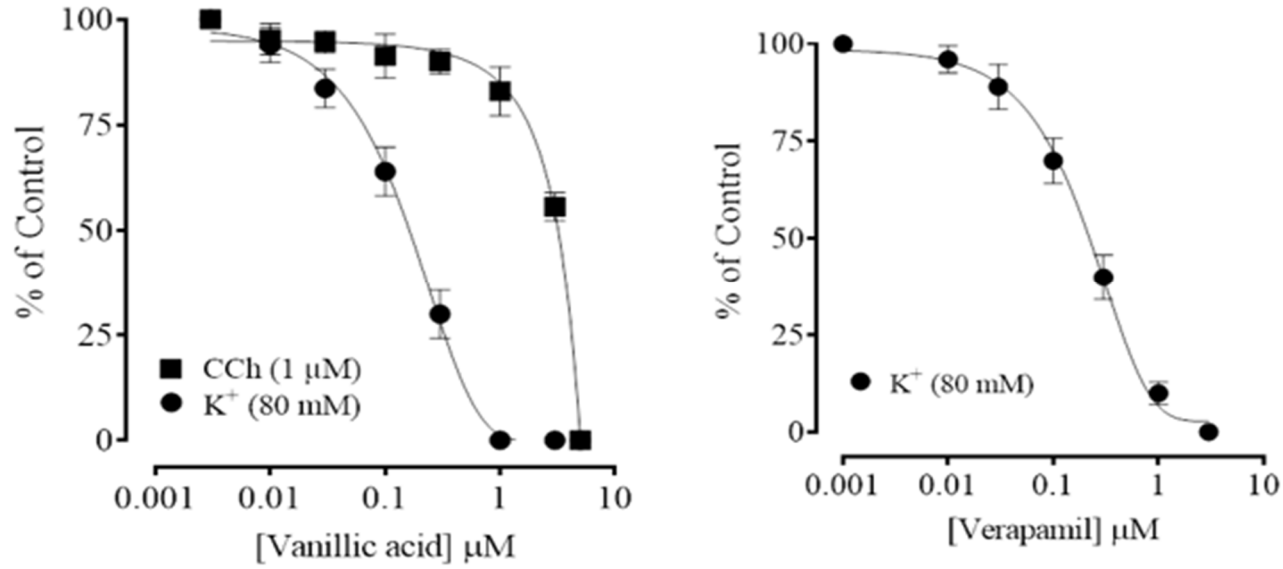

Fig. 6: Graph showing concentration response curve of (A) Vanillic acid against K^+ (80mM) and carbachol (CCh)-induced contractions (B) Verapamil against K^+ (80mM)-induced contraction in ileum preparations. Mean ± SEM (n=5).

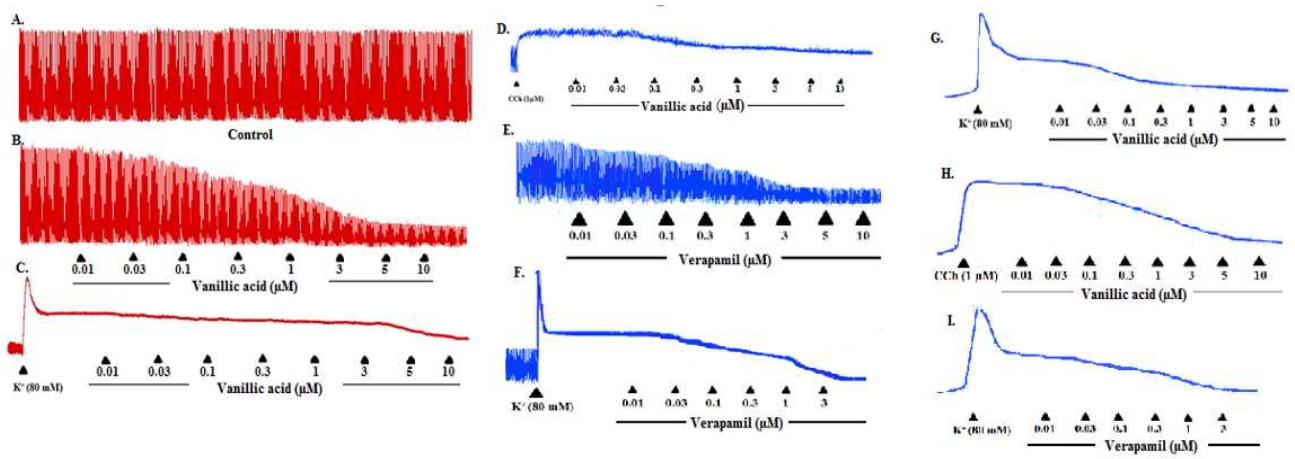

Fig. 7: Typical tracings showing (A) Control, the spasmolytic effect of vanillic acid against (B) Spontaneous, (C) K^+ (80 mM), (D) Carbachol (CCh:1μM)-induced concentrations, verapamil against (E) Spontaneous, (F) K^+ (80 mM) induced contractions against isolated mice jejunum preparations, vanillic acid against (G) K^+ (80mM), (H) Carbachol (CCh:1 μM), verapamil against (I) K^+ (80 mM)-induced contractions on isolated mice ileum preparations.

Table 1: Effect of vanillic acid and loperamide on castor-oil induced diarrhea in mice.

Treatment	Dose	Total no. of feaces in 4 h	Total no. of dry feaces in 4 h	Total no. of wet feaces in 4 h	% Protection = (mean number of dry feces/mean defecation) × 100
Vehicle	10 mL/kg	4.20 ± 0.20	4.09 ± 0.16	0.11 ± 0.05	97.50 ± 1.12
Castor oil	10 mL/kg	10.60 ± 0.24	0.40 ± 0.10	10.20 ± 0.20	3.70 ± 0.93
Vanillic acid	1 mg/kg	11.20 ± 0.49	1.60 ± 0.24	9.60 ± 0.68	14.01 ± 1.62*
	3 mg/kg	7.80 ± 0.37	1.60 ± 0.25	6.20 ± 0.37	25.84 ± 1.20*
	5 mg/kg	6.70±0.66	2.80 ± 0.20	3.40 ± 0.51	47.14 ± 1.75**
	7 mg/kg	5.40 ± 0.24	3.34 ± 0.16	2.06 ± 0.10	61.80 ± 0.92***
	10 mg/kg	5.30 ± 0.34	4.36 ± 0.17	0.94 ± 0.16	79.20 ± 0.49***
Loperamide	10 mg/kg	4.80 ± 0.37	3.96 ± 0.29	0.84 ± 0.09	82.67 ± 1.13***

Mean ± SEM (n = 5).
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. castor oil treatment, One way ANOVA followed by Dunnett's test.

Table 2: The antisecretory effect of vanillic acid on castor oil induced intestinal fluid accumulation in mice.

Treatment	Dose	Intestinal Fluid Accumulation *Pi*/*Pm* x 1000
Vehicle	10 mL/kg	105.5 ± 0.61
Castor oil	10 mL/kg	151.0 ± 0.63 #
Vanillic acid	1 mg/kg	142.2 ± 1.09*
	3 mg/kg	132.1 ± 0.71*
	5 mg/kg	124.1 ± 1.19**
	7 mg/kg	119.4 ± 0.63**
	10 mg/kg	111.7 ± 1.38***
Loperamide	10 mg/kg	106.3 ± 0.42***

Mean ± SEM (n=5).
#$p < 0.05$ vs vehicle, *$p < 0.05$,**$p < 0.01$, ***$p < 0.001$ vs. castor oil. One way ANOVA followed by Dunnett's-test.

Table 3: % Reduction in weight of intestinal content by vanillic acid in castor oil induced fluid accumulation in mice.

Treatment	Dose	Volume of intestinal content (mL)	% Reduction [(A-B)/A]×100	Weight of intestinal content (g)	% Reduction [(A-B)/A]×100
Vehicle	10 mL/kg	0.14 ± 0.01	NA	0.26 ± 0.02	NA
Castor oil	10 mL/kg	0.89 ± 0.02$^{\#}$	NA	0.94 ± 0.04$^{\#}$	NA
Vanillic acid	1 mg/kg	0.77 ± 0.02*	13.48	0.87 ± 0.03*	7.44
	3 mg/kg	0.52 ± 0.03*	41.57	0.59 ± 0.05*	37.23
	5 mg/kg	0.38 ± 0.03**	57.30	0.48 ± 0.03**	48.93
	7 mg/kg	0.31 ± 0.02**	65.16	0.34 ± 0.05**	63.82
	10 mg/kg	0.25 ± 0.03***	71.91	0.28 ± 0.06***	70.21
Loperamide	10 mg/kg	0.15 ± 0.01***	83.14	0.27 ± 0.01***	71.27

Mean ± SEM (n=5).
$^{\#}p < 0.05$ vs. vehicle, $^{*}p < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.001$ vs. castor oil. One way ANOVA followed by Dunnett's-test.

Table 4: Effect of vanillic acid on $_{L}$-arginine and PGE_2- induced enteropooling in mice.

Induction	Treatment	Dose	Intestinal Fluid (g)	Inhibition (%)
$_{L}$-arginine (600 mg/kg, p.o.)	Vehicle	10 mL/kg	0.89 ± 0.01	NA
	Vanillic acid	10 mg/kg	0.87 ± 0.07	2 %
	$_{L}$-NAME	10 mg/kg	0.63 ± 0.09*	30 %
PGE_2 (100 µg/kg, i.p.)	Vehicle	10 mL/kg	0.56 ± 0.04	NA
	Vanillic acid	10 mg/kg	0.40 ± 0.03*	29 %
	Loperamide	10 mg/kg	0.34 ± 0.03*	39 %

Mean ± SEM (n=5).
$^{\#}p < 0.05$ vs. vehicle, $^{*}p < 0.05$ vs. vehicle. One way ANOVA followed by Dunnett's-test.

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Author's contributions
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All data generated during and/or analyzed during this current study is included in this published article.

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The experiments performed comply with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC, 1996) and research ethics committee of COMSATS University Islamabad, Abbottabad Campus approved this project and provided the ethical approval number PHM. Eth/CS-M02-10-1124.

Conflict of interest
The authors declare no conflict of interest.

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