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Evaluation of *Medicago sativa* ethanol leaf extract for antidiarrheal activity in Wistar rats

Anele Donatus Onyebuchi [1], Akuodor Godwin Christian [2, *], Okoroama Casmir Emeka [2], Unaeze Bright Chukwuebuka [3], Ajegi Innocent Fountain [2], Iganga Ogbonnaya Nnabuike [4] and Nwokike Matthew Onyemaechi [5, 6]

[1] *Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Gregory University, Uturu, Abia State, Nigeria.*
[2] *Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Sciences, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Nigeria.*
[3] *Department of Medical Laboratory Science, Faculty of Health Science and Technology, Nnamdi Azikiwe University, Nnewi Campus, Nigeria.*
[4] *Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Sciences, Alex Ekwueme Federal University, Ndufu Alike Ebonyi State, Nigeria.*
[5] *Department of Pharmacology, Prince Abubakar Audu University, Anyigba, Kogi State, Nigeria.*
[6] *Department of Pharmacology, School of Medicine, Kabale, University, Uganda.*

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Abstract

Aim: *Medicago sativa* leaf is traditionally used for the treatment of gastrointestinal disorders in Nigeria. This research investigates the *in vivo* antidiarrheal activity of *M. sativa* (Alfalfa) leaf extract in Wistar rats.

Method: The study employed three models: castor oil-induced diarrhea, gastrointestinal motility (charcoal meal), and castor oil-induced intestinal fluid accumulation. The phytochemical analysis as well acute toxicity tests were carried out in the leaf extract.

Results: The results demonstrate a dose-dependent and significant antidiarrheal effect of *M. sativa* leaf extract. In the castor oil-induced diarrhea model, the extract reduced fecal frequency, delayed onset, and lowered severity, with the highest effect at 600 mg/kg compared to a positive control (Loperamide). Gastrointestinal motility was inhibited by the extract in a dose-dependent manner, achieving maximum effect at 600 mg/kg, comparable to atropine sulfate. The castor oil-induced intestinal fluid accumulation model revealed a significant decrease in fluid volume at 600 mg/kg, exhibiting a potent inhibitory effect. The oral LD50 values obtained were greater than 5000 mg/kg in rats

Conclusion: This study provides compelling evidence of *Medicago sativa* leaf extract's potential as an antidiarrheal agent in Wistar rats. Further investigations could explore its mechanism of action and safety profile, contributing valuable insights to the development of novel antidiarrheal therapies.

Keywords: *Medicago sativa*; Ethanol leaf extract; Antidiarrheal activity; Gastrointestinal motility; Enteropooling; Wistar rats

* Corresponding author: Akuodor Godwin Christian

1. Introduction

Diarrheal diseases remain a significant global health concern, particularly in developing countries, contributing to substantial morbidity and mortality rates, especially among children. The World Health Organization (WHO) estimates that diarrhea is responsible for approximately 1.6 million deaths annually, making it one of the leading causes of death in children under five years old [1]. The condition is often associated with microbial infections, ingestion of contaminated water or food, and various other factors compromising gastrointestinal health. Antidiarrheal agents play a crucial role in managing and preventing diarrhea, providing relief from symptoms and reducing the impact on overall health. Traditional medicinal plants have been a valuable source of natural compounds with therapeutic potential, including those possessing antidiarrheal properties. *Medicago sativa*, commonly known as Alfalfa, has been traditionally used in folk medicine for various health conditions, and there is a growing interest in exploring its pharmacological activities.

Medicago sativa is a perennial herb known for its rich nutritional content and potential health benefits. Previous research has reported its anti-inflammatory, antioxidant, and antimicrobial properties [2, 3]. However, the specific antidiarrheal effects of *Medicago sativa*, particularly in vivo, have not been comprehensively explored.

Previous studies have demonstrated the effectiveness of plant extracts in managing diarrhea through mechanisms such as reducing intestinal motility, inhibiting fluid accumulation, and exerting anti-inflammatory effects [4, 5]. Understanding the potential antidiarrheal mechanisms of *Medicago sativa* could contribute valuable insights to the development of novel therapeutic agents.

In this context, the proposed research aims to conduct an in-depth evaluation of the antidiarrheal activity of *Medicago sativa* leaf extract in Wistar rats. The study will involve the extraction of active compounds from *Medicago sativa* leaves, administration of different doses to rat models, and assessment of its impact on various diarrhea-induced parameters.

The choice of Wistar rats as an animal model is based on their well-established use in pharmacological research due to their physiological and genetic similarity to humans. The study design, including the use of castor oil-induced diarrhea models and gastrointestinal motility assessments, aligns with established methodologies for evaluating antidiarrheal properties [6, 7].

This research is expected to provide scientific evidence supporting the traditional use of *Medicago sativa* in managing diarrhea and could potentially contribute to the development of new antidiarrheal agents derived from natural sources.

2. Material and methods

2.1. Plant Collection and Identification

Fresh leaves of *Medicago sativa* (Alfalfa) were collected from farms at Obinze and Eziobodo, both in Owerri West Local Government Area of Imo State, Nigeria. The plant was identified and authenticated by Mr. Ibe Ndukwe, a taxonomist in the Department of Forestry, College of Environmental Sciences, Michael Okpara University of Agriculture, Umudike, Abia State and a sample specimen MOUAU/ZEB/21/009 was deposited in the University herbarium for reference.

2.2. Extraction of Plant Materials

The leaves were sliced into pieces, air-dried at room temperature and pulverized into powder using a Warring commercial blender. Eight hundred grams (800 g) of the coarse powder of *M. sativa* leaves were weighed by a sensitive digital weighing balance. The powder was soaked in a flask containing 80% ethanol (2.5 L w/v) and then placed on a shaker with occasional shaking for 48 hours at room temperature. The mixture formed was filtered using Whatman (No.1) filter paper and the filtrate was concentrated using a rotary evaporator and dried on a water bath. The percentage yield of the extract was determined, and the extract was labeled appropriately and stored at 4°C until further use. The extract was later reconstituted in distilled water to give desired doses of 200 mg/kg, 400 mg/kg, and 600 mg/kg body weight. The percentage yield was calculated using the formula of Ezirim *et al*. [8] as stated below:

$$\% \text{ yield} = \frac{\textbf{Weight(g) of residue (dry extract)}}{\textbf{Weight(g) of powdered material grinded}} \times \frac{\textbf{100}}{\textbf{1}}$$

2.3. Experimental Animals

Ninety (90) Adult Wistar rats (200 – 250 g) of either sex were used in this study. The animals were sourced from Department of Zoology, Faculty of Biological Sciences, University of Nigeria, Nsukka. They were kept in the animal house of the Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Abia State University, Uturu, Abia State. The animals were acclimatized for 14 days prior to the experiment. They were fed with standard diet (Ladokun feeds, Ibadan) and had access to water *ad libitum*. They were maintained under standard conditions of humidity, temperature and 12 hours light and 12 hours darkness cycle. The animals were used in accordance with the National Institute of Health guide for the care and use of Laboratory Animals [9].

2.4. Acute toxicity tests

This was determined following the method described by Lorke [10]. The study was carried out in two phases. In the first phase, nine rats were divided into three groups of three rats each. They were given 10 mg/kg, 100 mg/kg and 1000 mg/kg of the leaf extract respectively. They were however monitored for signs of toxicity initially for first 4 hours, and then for 24 hours. The signs of toxicity that were looked for include hyperactivity, paw licking, respiratory distress, and mortality. At the end of the first phase, there was no mortality. The study then proceeded to the second phase. In this phase, four rats were grouped into four with one rat in each group, and given 10 mL/kg distilled water, 1600 mg/kg, 2900 mg/kg and 5000 mg/kg of the extract respectively, and then monitored for signs of toxicity as stated earlier. The animals were further monitored for 48 and 72 hours for signs of late toxicity.

2.5. Phytochemical analysis

The method as described by Inyang et al., [11], Aziz, [12) was adopted for the phytochemical analysis of the ethanol extract of *M. sativa* leaf. The metabolites that were assayed include tannins, saponins, alkaloids, flavonoids, terpenoids, steroids, anthraquinones, glycosides, reducing sugars and resins.

2.6. Determination of anti-diarrheal activity

2.6.1. Castor oil-induced diarrheal model

The procedure outlined by Akuodor *et al.* [13] was used for the test. Thirty Wistar rats were used for this test and were divided in five groups of six rats each. They were kept at 25°C room temperature was fasted for 24 hours but had access to water prior to the commencement of the experiment. Each animal (rat) was placed in a separate cage with blotting paper lined on the floor. Group A were untreated and served as the control group, groups B, C and D were orally administered 200 mg/kg, 400 mg/kg, and 600 mg/kg of the *M. sativa* extract respectively, and group E was exposed to 4 mg/kg of Loperamide (positive control). After 30 minutes of treatment, each of the rats was orally given 1 mL of castor oil. The severity of diarrhea was monitored for a period of 5 hours. The total number of diarrhea (drops) was recorded and compared with the controls. The percentage (%) inhibition of diarrhea was calculated following the formula:

$$\% \text{ Inhibition of defecation} = \frac{A-B}{A} \text{ x } 100$$

Where *A* is the mean number of defecations caused by castor oil, and *B* is mean number of defecations caused by drug or extract.

2.6.2. Gastrointestinal Motility Model (Charcoal Meal)

The method described by Obiora et al., [14] was used. Fasted rats (as previously described) were divided into five groups of six rats in each cage. The control group received 10 mL/kg of distilled water orally. Animals in the second, third, and fourth groups received 200 mg/kg, 400 mg/kg, and 600 mg/kg of the fraction, respectively, whereas those in the positive control received atropine sulphate (5 mg/kg). After 30 min, the animals were all given 1 mL of charcoal meal (10% charcoal suspension in 5% tragacanth) orally. After 30 minutes, the animals were sacrificed by cervical dislocation, the abdomen was opened and the small intestine was isolated immediately. The small intestine of each animal was excised and the distance travelled by the charcoal meal was expressed as a percentage of the length of the small intestine according to the expression:

$$\% \text{ Inhibition } = \frac{\text{Mean length of intestine} - \text{Mean distance traveled by meal}}{\text{Mean length of intestine}} \times 100$$

2.7. Casto2.6.3 r oil-induced intestinal fluid accumulation model

The procedure described by Oyindamola et al [15] was adopted in the castor oil-induced enteropooling study. The test animals were deprived of food for 24 hours prior to the study. They were assigned into five groups of six animals each. Animals in the positive and negative control groups orally received 4 mg/kg of Loperamide and 20 mL of distilled water, respectively, whereas those in the test groups respectively received 200 mg/kg, 400 mg/kg, and 600 mg/kg of the leaf extracts. Immediately after these administrations, 1 mL of castor oil was administered orally to each rat in all the groups. After 30 minutes, the rats were sacrificed. The small intestine was excised and the intestinal contents were milked quantitatively into a measuring cylinder to obtain the volume and *mass* of the intestinal content.

The percentage of inhibition of intestinal content was determined by using the following formula.

$$\% \text{ Inhibition of intestinal fluid} = \frac{\text{Control} - \text{Test Extract}}{\text{Control}} \times 100$$

2.8. Statistical Analysis

Results were expressed as means ± SEM and analyzed with statistical products and services solution (SPSS version 20) by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A difference in the mean $P < 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical screening

Phytochemical screening of the leaf extract revealed the presence of tannins, saponins, flavonoids, steroids terpenoids, alkaloids, and cardiac glycosides, while phlobatannins and phenol were absent.

3.2. Acute toxicity test

The oral acute toxicity test showed normal behaviour of rats treated the leaf extract of *M. sativa*. There were no lethality or toxic reactions observed. However, the experimental doses used, 200 mg/kg, 400 mg/kg and 600 mg/kg were orally within safe margin.

3.3. Effect of *M. sativa* on castor oil-induced diarrhea in rats

The 200 mg/kg, 400 mg/kg, and 600 mg/kg of the ethanol leaf extract of *M. sativa* produced a dose-dependent and significant ($P < 0.05$) protection of rats against castor oil-induced diarrhea, which led to the decreased number of feces. The ethanol leaf delayed the onset of castor oil-induced diarrhoea, decreased the frequency of defecation (number of wet feces and total number of feces), and reduced the severity of diarrhoea in rats. Loperamide (4 mg/kg), a standard antidiarrhoeal drug produced a significant inhibitory effect ($p<0.01$) (Table 1 and Fig. 1).

3.4. Effect of *M. sativa* on intestinal transit in rats

The oral administration of the leaf extract produced dose-dependent and significant ($P < 0.05$) effect and slowed the propulsive movement and transit charcoal meal. The atropine sulphate at 5 mg/kg showed more anti-motility effect than 400 mg/kg of the leaf extract used. However, the maximum effect of the leaf extract was achieved at 600 mg/kg (Table 2 and Fig. 2).

3.5. Effect of *M. sativa* on castor oil-induce- intestinal fluid accumulation

The ethanol leaf extract of *M. sativa* dose - dependently and significantly ($P < 0.05$) decreased the volume of intestinal fluid with corresponding increase in inhibition of the intestinal fluid content in the castor oil-induced intestinal fluid accumulation model. The highest effect on the volume of intestinal content was achieved at 600 mg/kg as shown in Table 3 and Fig. 3.

Table 1 Effect of *M. sativa* ethanol leaf extract in castor oil induced diarrhoea in rats

Drug	Dose(mg/kg)	Mean diarrhea frequency (4 h)	% Inhibition
Control	0.2 mL	12.17±0.87	**0.0**
M. sativa	200 mg/kg	4.33±0.67	64[a]
M. sativa	400 mg/kg	2.80±0.65	77[b]
M. sativa	600 mg/kg	1.33±0.56	89[b]
Loperamide	4 mg/kg	1.00±0.52	92[b]

One-way ANOVA + Dunnett's post hoc test (n=6). [a] P<0.05, [b] P<0.01 compared to control.

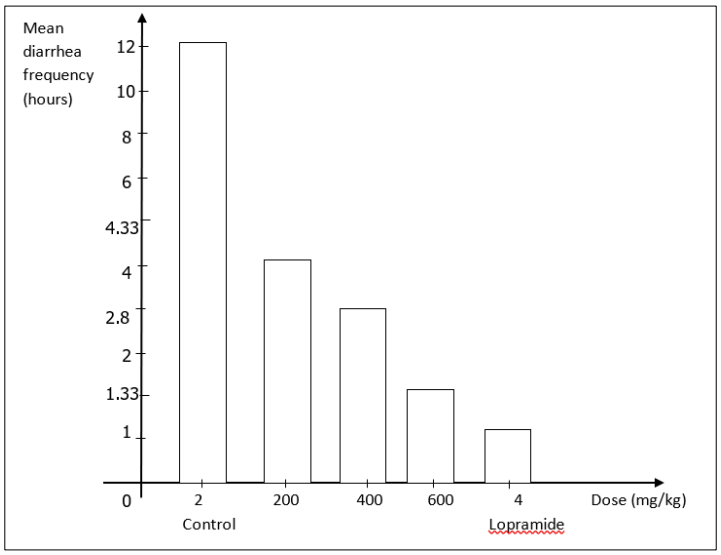

Figure 1 Bar Chart of *M. sativa* extract and castor oil induced diarrhea

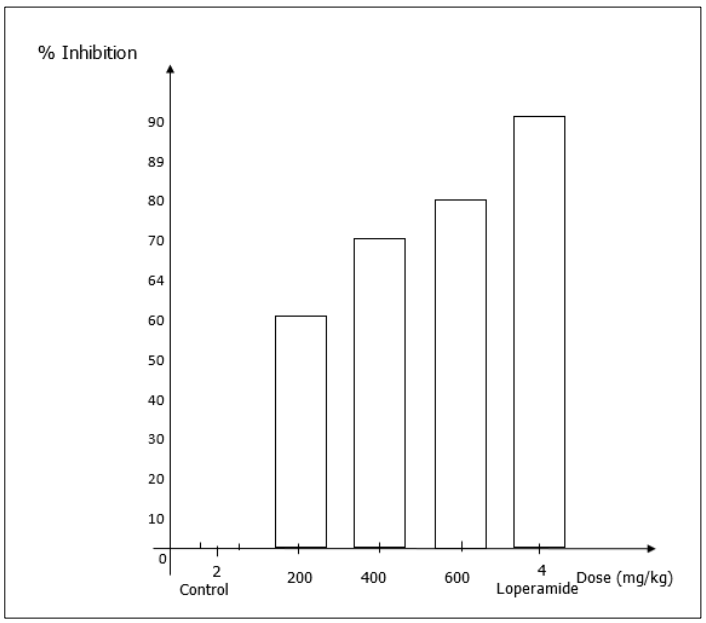

Figure 2 Bar Chart of *M. sativa* extract and % inhibition of castor oil induced diarrhea

Table 2 Effect of *M. sativa* ethanol leaf extract on intestinal transit time in rats

Drug	Dose(mg/kg)	Mean intestinal length (cm)	Distance covered by charcoal meal (cm)	% Inhibition
Control	0.2 mL	88.83±1.81	86.17±2.07	0.0
M. sativa	200 mg/kg	86.50 ± 2.	46 27.17±2.07	70[b]
M. sativa	400 mg/kg	86.00 ± 1.88	21.17 ± 2.75	75[b]
M. sativa	600 mg/kg	82.50 ± 1.84	13.50 ± 1.35	84[b]
Atropine	5 mg/kg	84.50±1.61	11.83±1.05	86[b]

One-way ANOVA + Dunnett's post hoc test (n=6). [b] P<0.01 compared to control

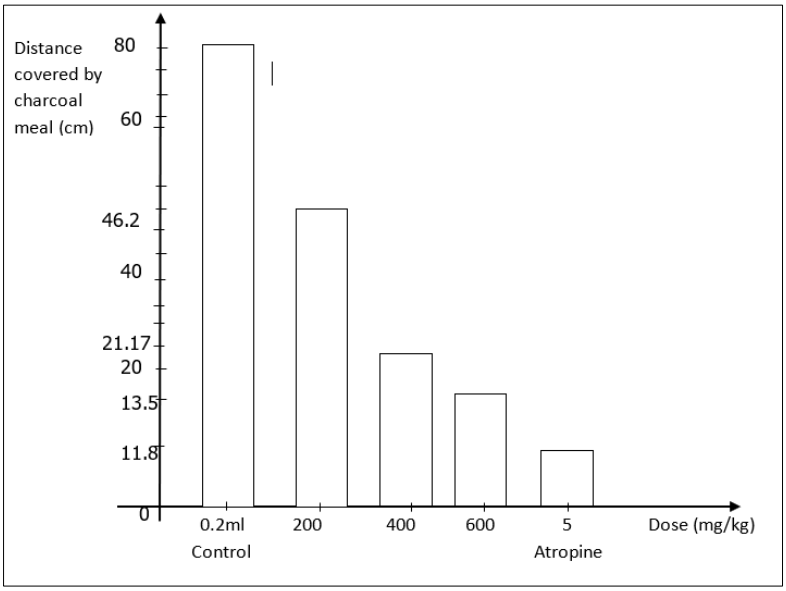

Figure 3 Bar Chart of *M. sativa* extract and intestinal transit (charcoal meal)

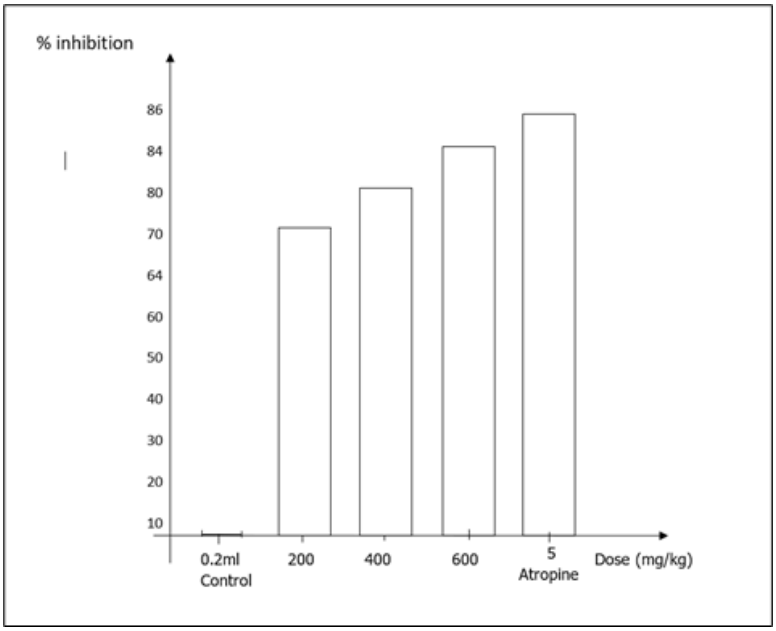

Figure 4 Bar Chart of *M. sativa* extract and % inhibition of intestinal transit (charcoal meal)

Table 3 Effect of M. sativa ethanol leaf extract on castor oil induced intestinal accumulation in rats (Enteropooling)

Drug	Dose (mg/kg)	Intestinal fluid (mL)	% Inhibition
Control	0.2 mL	3.70±0.08	0.0
M. sativa	200 mg/kg	1.35 ± 0.03	64[a]
M. sativa	400 mg/kg	0.87 ± 0.18	77[b]
M. sativa	600 mg/kg	0.42 ± 0.18	87[b]
Loperamide	4 mg/kg	0.37 ± 0.12	90[b]

One-way ANOVA + Dunnett's post hoc test (n=6). [a] P<0.05, [b] P<0.01 compared to control.

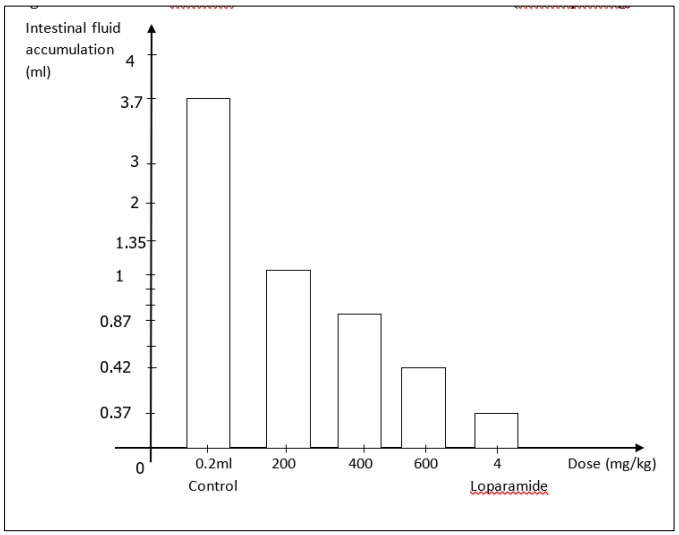

Figure 5 Bar Chart of *M.sativa* extract and intestinal accumulation (Enteropooling)

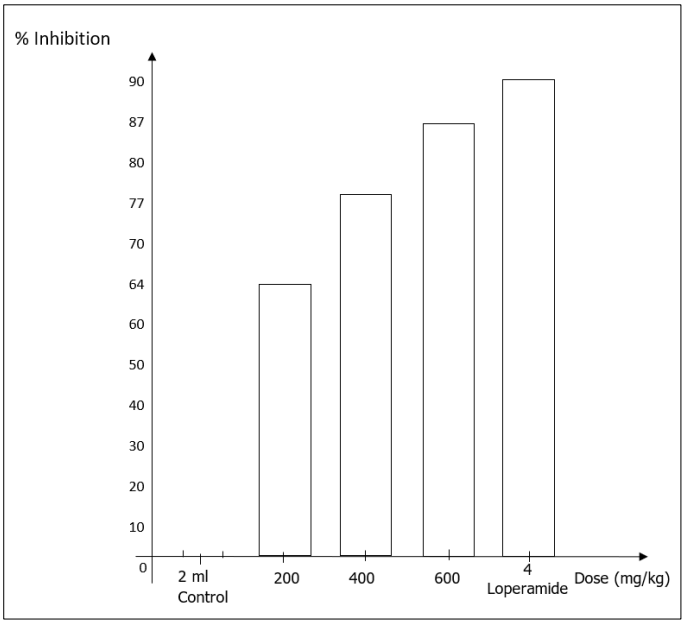

Figure 6 Bar Chart of *M. sativa* extract and % inhibition of intestinal accumulation (Enteropooling)

4. Discussion

Diarrhea is one of the most common and serious diseases in almost all tropical countries of the world, is a principal cause of morbidity and mortality as a result of fluid loss and severe dehydration among children in developing countries [16]. Despite the availability of a vast spectrum of orthodox drugs for diarrheal control, the majority of people in many developing countries still rely on herbal agents for the management of the disease [17]. Many plants in Nigeria have been reported to be effective against diarrhea and dysentery as they were used by local communities as traditional folklore medicine.

Castor oil was used in three models in this study to induce diarrhea. This chemical induced diarrhea through a pathophysiological mechanism may be caused by its active metabolite(s). Castor oil is known to be metabolized into recinoleic acid, a hydroxylated fatty acid, by the action of intestinal lipases in the intestinal lumen and considerable amounts of recinoleic acid was absorbed in the intestine [18]. The release of recinoleic acid resulted in the irritation and inflammation of intestinal mucosa, leading to the liberation of inflammatory mediators viz histamine, prostaglandins, and others [19]. The metabolize initiated diarrhea by binding with EP3 prostanoid receptors on smooth muscle cells. However, the liberated prostaglandings promoted vasodilation, mucus secretion and contraction of the smooth muscles in the small intestines. The prostaglandin E series is known to be diarrhoegenic in both laboratory animals and in humans. Ricinoleic acid is shown to increase sodium and water secretion through its ultrastructural alterations in the villous tips of the intestinal mucosa to bring about diarrhea as observed in these animals of this study [20, 21]. Other studies indicated that hydroxyfatty acids (including ricinoleic acid) include fluid and electrolyte secretion secondary to their stimulation of an active anion secretory process [22]. Normal intestinal fluid absorption was also impaired by castor oil through inhibition of intestinal Na^+/K^+ ATPase activity [23]. In addition, castor oil (ricinoleic acid) also altered the motility of GI smooth muscles [24]. Therefore, the use of castor oil as diarrhea inducer for all models was plausible as it miniimized the pathophysiologic processes. Ricinoleate stimulated endogenous secretion of the prostaglandin [25].

Disruption of prostaglandin biosynthesis might shorten castor oil induced diarrhea. It was also documented vividly that loperamide (the standard drug used as antidiarrheal agent in this study), antagonized the diarrhea induced by castor oil and these actions were due to anti-secretory and antimotility properties [26].

In the gastro intestinal motility test model, the ethanol leaf extract of M. Savita reduced gastrointestinal motility in castor-oil-treated rats as shown by the reduction in gastrointestinal movement of charcoal meal. The extract had been shown to decrease the intestinal fluid accumulation [19]. This suggested that the plant leaf extract might decrease water and electrolyte secretion to the intestinal lumen while promoting their absorption, which in turn could decrease intestinal overload and distension, leading to decrease in intestinal motility (longer time for absorption) and water contents of the feacal drugs, hence overall reduction in the total number of defecation instances and diarrheal drops in treated groups. This was consistent with the mechanism of action of Loperamide for its antidiarrheal effect as presented in literatures [27.

Furthermore, the extract was reported to possess anti-cholinergic activity and caused reduction in intestinal motility and secretion which was in agreement with the action of atropine on the intestine [28]. Charcoal passage test was commonly used to determine the effect of the test substances on gut motility. Atropine block M1 receptors on gastric parietal cells and helped in reduction of gastric secretions. It also blocked M3 receptors on visceral smooth muscles and decreased the tone and amplitude of these organs [29].

Hence, atropine was used as standard antisecretory drug for comparism in charcoal passage test. The ethanol leaf extract in the castor oil induced GI motility test significantly showed down charcoal meal transit in GIT, thus leading to relaxation of the gut muscles and showing down motility [30]. This assumption was further supported by the antispasmodic of the leaf extract of this plant by antagonizing the actions of acetylcholine [31]. The presence of Tannins in the leaf extract had been demonstrated to inhibit GI movement by reducing the intracellular Ca^{2+} inward current or by activation of the calcium pumping system [32] as well as by forming protein tannates which made the intestinal mucosa resistant and hence reduced peristaltic movement [33]. The presence of terpenoids in the leaf extract demonstrated antispasmodic activity and had an inhibitory effect on GI motility [34, 35].

In castor oil induced intestinal fluid accumulation study, the leaf extract demonstrated significant reductions of water contents at different doses, as well as frequency of defecation and intestinal fluid accumulation. The remarkable inhibition of castor oil induced intestinal fluid in rats, showed that the extract might produce relief in diarrhea through its spasmolytic and against intestinal fluid accumulation effects. Similar to the findings in the castor oil – induced diarrhea model, all doses of the leaf extract showed significant reduction in the volume of intestinal fluid compared with

the negative controls. The percentage reduction in volume of intestinal content was increased with the dose of the extract. This result demonstrated that the effect of this plant's leaf extract on percent inhibition of castor oil-induced intestinal fluid accumulation is increased as its doses increased. The results revealed that the effect of the highest dose of the leaf of intestinal fluid accumulation was found to be closer to and comparable with the inhibitory effect of Loperamide.

The findings in this study might indicate that the leaf extract has significant antisecretory effect and this contributed to its antidiarrheal effect as seen in castor oil-induced diarrhea model.

Reports in literatures showed that terpenoids, flavonoids and tannins inhibit the active secretion of ricinoleic acid, resulting in the activation of Na^+, K^+ ATPase activity that promotes absorption of Na^+ and K^+ in the intestinal mucosa [36] which is linked with a decrease in frequency of feaces. They are shown to promote colonic absorption of water and electrolytes [34].

Studies showed that ricinoleic acid might activate the nitric oxide pathway and induce nitric oxide dependent gut secretion [37]. Other studies also confirmed that NO was involved in the causation of diarrhea and this was counteracted by agents that inhibit NO synthesis [31].

In this study, the ethanol leaf extract of *Medicago sativa* had been shown to inhibit castor oil-induced diarrheal episodes, intestinal secretion and motility which might be produced by the phytochemical constituents. These results could be taken as scientific evidence that the leaf of this plant had strong antidiarrheal activity. Also, this plant was found to be safe, as no sign of toxicity was noted in the acute oral toxicity test which indicates that the plant was tolerable and safe.

5. Conclusion

Medicago sativa leaf extract showed promising antidiarrheal effects in Wistar rats, suggesting its potential therapeutic use. Further studies are warranted to elucidate the underlying mechanisms and evaluate its safety and efficacy in humans, contributing valuable insights to the development of novel antidiarrheal therapies.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Compliance with ethical standards

Statement of ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Also, all procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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