WORLD JOURNAL OF GASTROENTEROLOGY - MANUSCRIPT NO: 56930

The authors must resolve all issues in the manuscript based on peer-review report(s) and make a point-to-point response to the issues raised in the peer-review report(s) which listed below:

REVIEWER 1:

Comment 1: The title should include only the demonstrated effects, not antimicrobial, as the effect was demonstrated only for fungi.

Answer: As requested by the reviewer, the title was modified: *"Antifungal and antidiarrheal activity via antimotility mechanisms of (-)-fenchone in experimental models"*.

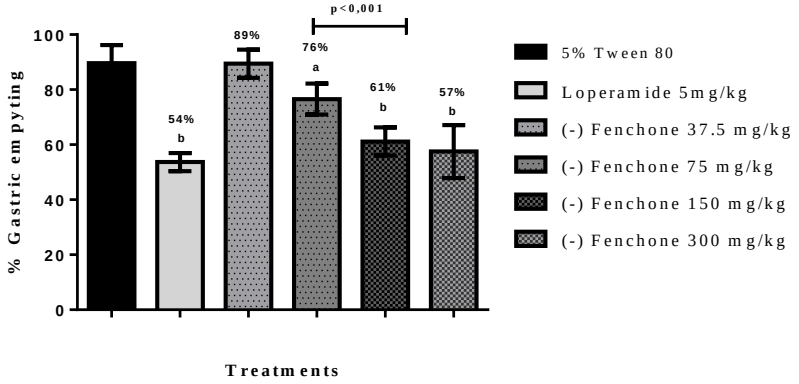
Comment 2: The background (introduction) includes most important aspects of diarrhea (etiology, treatment, limitation of current methods and need for searching new alternatives). Many recent study tries to find if different species of plant compound could be used as treatment of gastrointestinal disorders. Material and methods are described correctly, the assays used give the possibility to evaluate the antidiarrheal activity (evacuation index, percentage of liquid stools and diarrhea inhibition), gastric emptying, intestinal transit using activated charcoal (better use this wore all the times, not activated carbon), antimotility action, antibacterial and antifungal activity.

Answer: All text has been modified to "*activated charcoal"*.

Comment 3: The results are presented in a convincing manner. I would change the figures 1, 2, 3 limiting the y-scale to 100%, as in figure 4 and 5.

Answer: We agreed with the reviewer statement, and Fig 1, 2, and 3 were modified, limiting the y-scale to 100%.

e.g., Figure 1 (represented below).



Comment 4: As the conclusions said, this compound has no antimicrobial activity and this should be emphasized, as more diarrhea cases are due to microbial agents than fungal agents.

Answer: The text has been modified to: *"Monoterpene inhibited the growth of fungal strains, being considered a product with strong antifungal activity, but without antibacterial activity."* Title, abstract, and body text have been changed to emphasize that (-)-fenchone does not have antibacterial activity, only antifungal.

Comment 5: There is a need of check for English language for fine changes. Also some sentences are too long (even one paragraph long - 8 lines - ex. Muscarinic receptor agonists...)

Answer: In agreement with the reviewer's considerations, the most extended paragraphs have been summarized in the text. For example, "*Acetylcholine (ACh) exerts an excitatory effect on the gastrointestinal smooth muscle by activating muscarinic M_3 receptors (coupled to $Gq/_{11}$), there is an increase in the cytosolic concentration of Ca^{2+}, which results in contraction of the smooth muscle and increased intestinal transit*".

The article went through some changes in writing, and the English language was verified.

REVIEWER 2:

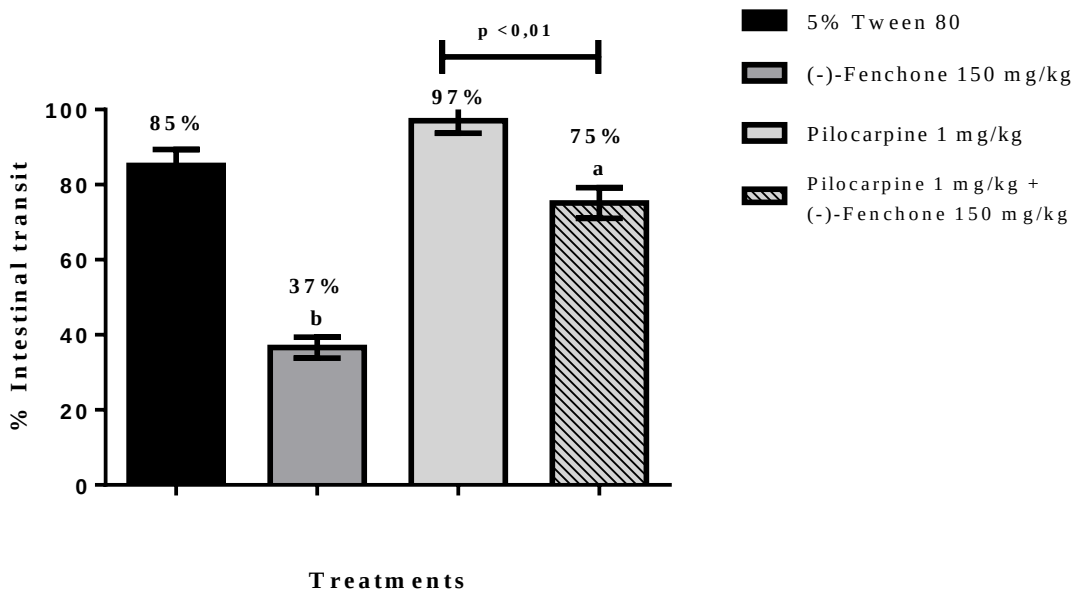
Comment 1. "In conclusion, the results of the present investigation indicate that (-)-fenchone has antidiarrheal activity, which is related to an antimotility effect, through the participation of muscarinic, adrenergic, nitrergic pathways and KATP channels, not being related to antisecretory or pro-absorptive activities." To be able

to confirm this statement very specific blockers or agonists should be used. The conclusion should be amended to say :(-)-fenchone has antidiarrheal activity, which is related to an antimotility effect. This antimotility effect can be blocked by α2 and β adrenergic receptor antagonists. It can also be blocked by L-Name indicating a possible role of NO. The sameapplies to glybenclamide.

Answer: We agree with the reviewer's opinion, and the conclusion has been changed to: *"(-)-fenchone presents antidiarrheal activity, related to an antimotility effect. This antimotility effect involves anticholinergic mechanisms, which can be partially reversed in the presence of a muscarinic agonist. It can be blocked by α2 and β adrenergic receptor antagonists, suggesting the participation of the adrenergic pathway. It can also be blocked by L-NAME and glibenclamide, indicating possible participation of NO and KATP channels."*

Comment 2. In the section: "Effect of oral administration of (-)-fenchone after treatment with pilocarpine and yohimbine and on intestinal transit of mice"". It is not clear if the difference between columns 1 and 4 (Pilocarpine and Pilocarpine+fenchone) is significant. If the difference is not significant it is not clear why the authors conclude that fenchone is acting via muscarinic receptors.

Answer: In the group pretreated with pilocarpine (non-selective muscarinic receptor agonist, 1 mg/kg i.p), fenchone significantly ($p<0,01$) reduced muscarinic action in intestinal transit to 75%, when compared with the group of animals that received only pilocarpine (muscarinic agonist - 1 mg/kg) in the absence of (-) - fenchone that presented a percentage of intestinal transit of 97%, suggests that the antimotility effect of fenchone is related to blocking this pathway. Figure 3 has been changed for better visualization of results, and statistical significance has been added. This result was better discussed in the text, in the following paragraph: *""Pilocarpine, a cholinergic agonist, was used to induce intestinal motility. It can be seen that fenchone significantly reduced the stimulating effects promoted by pilocarpine resulting in reduced intestinal transit. From this result, we can infer that (-)-fenchone may be competing for GIT M_3 receptors, acting as a partial antagonist of muscarinic receptors and that its antimotility involves the cholinergic pathway".*



Comment 3. The effect of pilocarpine is long lasting, in that group of mice were the animals exposed to pilocarpine once or twice?

Answer: Pilocarpine was used to evaluate the participation of muscarinic receptors in the antimotility effect exerted by (-) - fenchone. This protocol was conducted according to the methodology standardized by Santos and Rao (1999). For this, the mice (submitted to a 24-hour fast) were divided into 4 different experimental groups: Negative control (vehicle), (-) - fenchone 150 mg / kg, pilocarpine 1 mg / kg and pilocarpine + fenchone. For both groups with pilocarpine, this substance was administered only once. In the pilocarpine + fenchone group, pilocarpine was administered 30 minutes before the administration of fenchone. After 60 minutes, the activated carbon marker was administered, and 30 minutes later, the animals were euthanized. The duration of the effects of pilocarpine is 4 to 8 hours.

(Santos FA, Rao VS. Quinine-induced inhibition of gastrointestinal transit in mice: possible involvement of endogenous opioids. Eur J Pharmacol 1999; 364: 193-197 [PMID: 9932723 DOI: 10.1016] https://pubchem.ncbi.nlm.nih.gov/compound/pilocarpine#section=Mechanism-of-Action
https://www.iberoquimica.com.br/Arquivos/Insumo/arquivo-163107.pdf)

Comment 4. The description of each experiment should be more detailed. Were the groups treated as paired?

Answer: These mechanisms were grouped because they share the same methodology (Santos and Rao, 1999). In this protocol for evaluating the antimotility mechanisms, a group of animals received pretreatment with a single dose of standard drugs. It has well-described mechanisms of action, interfering with the different signaling pathways (pilocarpine - 1 mg/kg; yohimbine - 1 mg/kg; propranolol - 1 mg/kg; L-NAME - 25 mg/kg; or glibenclamide 1 mg/kg) intraperitoneally, which were treated in parallel with the

groups that received only 0.9% NaCl as pretreatment. After 30 minutes of pretreatment, the animals received orally, the vehicle (tween 80 5%) or (-) - fenconha (150 mg/kg), according to the group belonging. The protocols were described in more detail, as can be seen below: *"Two groups received intraperitoneal NaCl solution 0.9% (10 mL/kg), the other two received pilocarpine (non-selective muscarinic receptor agonist, 1 mg/kg), yohimbine (α_2-adrenergic presynaptic receptor antagonist, 1 mg/kg), propranolol (non-selective β adrenergic receptor antagonist), L-NAME (NO synthase activity inhibitor, 25 mg/kg) or glibenclamide (K_{ATP} channel blocker, 1 mg/kg). These drugs were dissolved in NaCl 0.9% and given intraperitoneally. After 30 min, the animals were treated orally with 5% tween 80 (control group), or fenchone 150 mg kg (most effective dose). After 60 min, 10 mL/kg (p.o.) of the black marker (10% activated charcoal suspension in 5% Arabic gum) was administered, and 30 min later, the animals were euthanized for removal of the small intestine to calculate the percentage of intestinal transit".*

Comment 5. In the Introduction, the authors state that "Among the main classes of drugs used are antisecretory and motility suppressing agents, probiotics, enkephalinase inhibitors, bismuth compounds, α2-adrenergic receptor agonists, and muscarinic agonist". Muscarinic agonists usually enhance motility and secretion. If the authors think that a subtype of inhibitory muscarinic agonist is involved, they should specify it in this statement. Also when describing the microbiota they mention that "changes in microorganisms can cause motility disorders." This is not well established yet and the reference is not relevant.

Answer: This section was revised, and muscarinic agonists were removed from the classes of drugs mentioned. The text has been modified to: *"Among the main classes of drugs used are antisecretory and motility suppressing agents, probiotics, enkephalinase inhibitors, bismuth compounds, and α_2-adrenergic receptor agonists"*. Concerning the microbiota, this theme has been studied more intensely in recent years. Some studies have shown the role of dysbiosis as a predisposing factor for the development of some diseases of the gastrointestinal tract, such as inflammatory bowel diseases, irritable bowel syndrome and diarrhea (Bin et al., 208; Hu et al., 2018; Yue et al., 2019; Zhu et al., 2018). However, it is not yet fully established. Thus, it was excluded from the manuscript, as suggested by the reviewer.

(Bin et al., 2018 - doi:10.1186/s12917-018-1704-9; Hu et al., 2018 - doi:10.1016/j.chom.2018.11.006; Yue et al., 2019 - doi:10.1016/j.biopha.2019.109002; Zhu et al., 2018 - doi:10.1264/jsme2.ME17163).

Comment 6. The description of the methodology for the assessment of the antibacterial effect of fenchone is lacking. The bacterial count or method of measurement should be described in detail.

Answer: The topic describing the methodology for assessing the antibacterial effect of (-)-fenchone has been revised, and the methods been described in detail. The text has been modified to *"For inoculum preparation, the colonies of microorganisms were suspended in 0.85% sterile 0.9% NaCl solution and adjusted according to the 0.5 scale of McFarland standard to obtain an inoculum of 1-5.106 colony-forming units per milliliter (CFU.L-1) for fungi and 1-2.108 CFU.mL-1 for bacteria. Antimicrobial activity assays*

were performed according to the protocols of Cleeland and Squires, Eloff, and CLSI. MIC determination of the (-)-fenchone on bacterial and fungal strains was performed by the microdilution technique in 96 well plates. Initially, 100 µL of double concentrated RPMI/BHI broth was distributed to the wells of the microdilution plates. Then, 100 µL of (-)-fenchonewas dispensed into the wells of the first line of the plate and by serial dilution at a ratio of two concentrations of 1024 µg/mL to 16 µg/mL were obtained. Finally, 10 µL of bacterial and fungal inoculums (strains of Staphylococcus aureus ATCC-25923 and LM-177, Pseudomonas aeruginosa ATCC-25853 and LM-297, Escherichia coli ATCC-18739 and LM-39, Candida albicans ATCC-76645 and LM-05; Candida tropicalis ATCC-13803 and LM-20; Candida Krusei ATCC-6258 and LM-13) were added to the wells. Controls performed: microorganisms and culture medium to check the viability of the strains and the sterility of the medium and control with Gentamicin (64 µg/mL) and amphotericin B (32 µg/mL). The prepared plates were sealed aseptically and incubated at 35 ± 2 ° C for 24-48 hours. The antimicrobial activity of the products was interpreted and considered as active or inactive according to the following criteria: up to 600 µg/mL = strong activity; 600-1500 µg/mL = moderate activity; > above 1500 µg/mL = weak activity or inactive product. After MIC, 10 µL aliquots of the supernatant from the wells in which complete fungal growth inhibition (MIC, MICx2, MICx4, and MICx8) was observed on the microdilution plates were added to 100 µL RPMI broth contained in new culture plates. Plates were incubated for 24-48 h at 35 ± 2 ° C. Minimum Fungicide Concentration (MFC) was considered as the lowest concentration of the product that was able to inhibit the growth of microorganisms".

Minor Comment 1. In the paragraph "Evaluation of the participation of muscarinic, adrenergic, nitrergic pathway and ATP-dependent potassium channels (KATP) in (-) fenchone antimotility mechanisms in the intestinal transit model" it is mentioned that after 30 min of "blockade" vehicle or fenchone is administered. It is not clear what "blockade" the authors are describing. The details of the steps of the experiments for each agent are lacking.

Answer: The protocols have been revised and described in more detail, and the manuscript has been modified, as shown below:

"To investigate the antimotility mechanisms involved in the antidiarrheal activity, fenchone were evaluated according to the model described by Santos and Rao. The role of muscarinic receptors, alpha and beta-adrenergic receptors, NO, and KATP were evaluated. The mice were fasted for 24 hours and distributed in different groups (n =7per group). Two groups received intraperitoneal NaCl solution 0.9% (10 mL/kg), the other two received pilocarpine (non-selective muscarinic receptor agonist, 1 mg/kg), yohimbine (α2-adrenergic presynaptic receptor antagonist, 1 mg/kg), propranolol (non-selective β adrenergic receptor antagonist), L-NAME (NO synthase activity inhibitor, 25 mg/kg) or glibenclamide (KATP channel blocker, 1 mg/kg). These drugs were dissolved in NaCl 0.9% and given intraperitoneally. After 30 min, the animals were treated orally with 5% tween 80 (control group), or fenchone 150 mg kg (most effective dose). After 60 min, 10 mL/kg (p.o.) of the black marker (10% activated charcoal suspension in 5% Arabic gum) was administered, and 30 min later, the animals were euthanized for removal of the small intestine to calculate the percentage of intestinal transit".

Comment 2. When describing the Results please describe the properties of each agent used (e.g., yohimbine is an α2 receptor antagonist).

Answer: Once a possible antimotility activity was verified, more mechanical tests were needed to elucidate the pathways involved in this effect. For this, we use drugs with well-known mechanisms that act in these pathways, such as pilocarpine (muscarinic agonist), yohimbine (α2 adrenergic antagonist), propranolol (non-selective β-adrenergic receptor antagonist), L-NAME (nitric oxide synthase inhibitor) and glibenclamide (KATP channel blocker). Description of the properties of each of the drugs used was made throughout the text of the article, in the sections of methodology, results, and discussion. For example: *""Pretreatment with L-NAME (inhibitor of NO synthase activity, 25 mg/kg ip) or glibenclamide (KATP channel blocker, 1 mg/kg i.p) reversed the inhibitory effect of this monoterpene on intestinal transit to 85 and 92%, respectively, when compared to the unblocked group (-)-fenchone".*

Comment 3. Figure legend: n=8, does this number indicate the number of animals in each group? specify and indicate the numbers for each group in all the figures.

Answer: Yes, this number indicates the number of animals in each group. It was added, *"n=7/per group"* in all legends. e.g., *"Figure 1. Effect of oral administration of (-)-fenchone and loperamide on gastric emptying in male Swiss mice. Data expressed as mean ± standard deviation and analyzed by ANOVA, followed by Dunnet and Tukey'sTukey's multiple comparison tests (aP<0.01 and bP<0.001 - compared to the 5% Tween 80 group) (n = 7/per group)."*

Comment 4. Activated charcoal (10%) in gum arabic 5%. The description is lacking for the remaining compounds.

The function of all the substances used has been described in the text. Gum arabic is used as a thickening agent, easily dissolves in hot water and produces less viscous solutions than carboxymethylcellulose (CMC), and can be used in concentrations of up to 10% as soluble fiber (Caleguer; Benassi, 2007).

(Caleguer; Benassi, 2007 - https://doi.org/10.1590/S0101-20612007000200010).

Comment 5. Please spell out abbreviations MIC, MCB, and MFC in the text

Answer: The abbreviations MIC and MFC have been described in the text, as can be seen below: minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The abbreviation MCB was excluded.

Comment 6. CaV-L should be written Cav(L)

Answer: The paragraph that talked about the CaV (L) channels was deleted at the suggestion of another reviewer to make the discussion more objective.

Comment 7. Referring to "test substance" is confusing because many compounds were used. Please indicate the name of the substance.

Answer: It has been changed to (-)-fenchone throughout the text.

Comment 8. "Then, 100 µL of the substance was dispensed into the wells of the first line of the plate and by serial dilution at a ratio of two concentrations of 1024 µg/mL to 16 µg/mL were obtained. Finally, 10 µL of the suspensions of strains were added to the wells." The substance should be defined as well as the "suspension of strains." Please clarify the methods for this experimente.

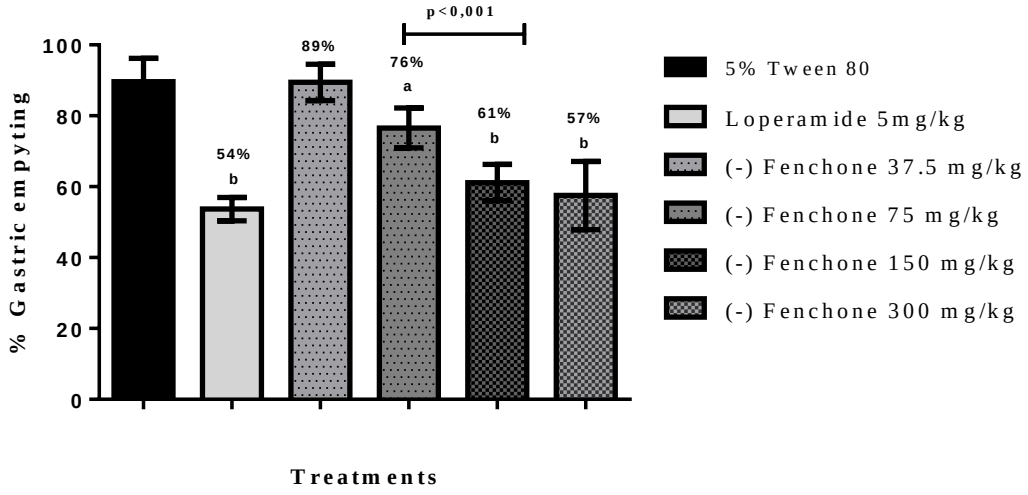
Answer: The text was changed to: *"The tests for antimicrobial activity were carried out according to the protocols of Cleeland and Squires, Eloff and Clinical Laboratory Standards Institute. The determination of the (-) - fenchone MIC in bacterial and fungal strains was carried out using the microdilution technique in 96-well plates. Initially, 100 µL of double concentrated RPMI / BHI broth were distributed to the microdilution plate wells. Then, 100 µL of (-) - fenchone were dispensed in the wells of the first row of the plate and by serial dilution, in the proportion of two concentrations of 1024 µg / mL to 16 µg / mL. Finally, 10 µL of bacterial and fungal inoculum was added to the wells. Controls performed: microorganisms and culture medium to check the viability of the strains and the sterility of the medium and control with Gentamicin (64 µg / mL) and amphotericin B (32 µg / mL). The prepared plates were sealed aseptically and incubated at 35 ± 2 ° C for 24-48 hours. The antimicrobial activity of the products was interpreted and considered active or inactive, according to the following criteria: up to 600 µg / mL = strong activity; 600-1500 µg / mL = moderate activity; > above 1500 µg / mL = weak activity or inactive product."* The strains used were: Bacterias *Staphylococcus aureus* ATCC-25923 and LM-177, *Pseudomonas aeruginosa* ATCC-25853, and LM-297, *Escherichia coli* ATCC-18739 and LM-39. Fungi *Candida albicans* ATCC-76645 and LM-05, *Candida tropicalis* ATCC-13803 and LM-20, *Candida Krusei* ATCC-6258 and LM-13.

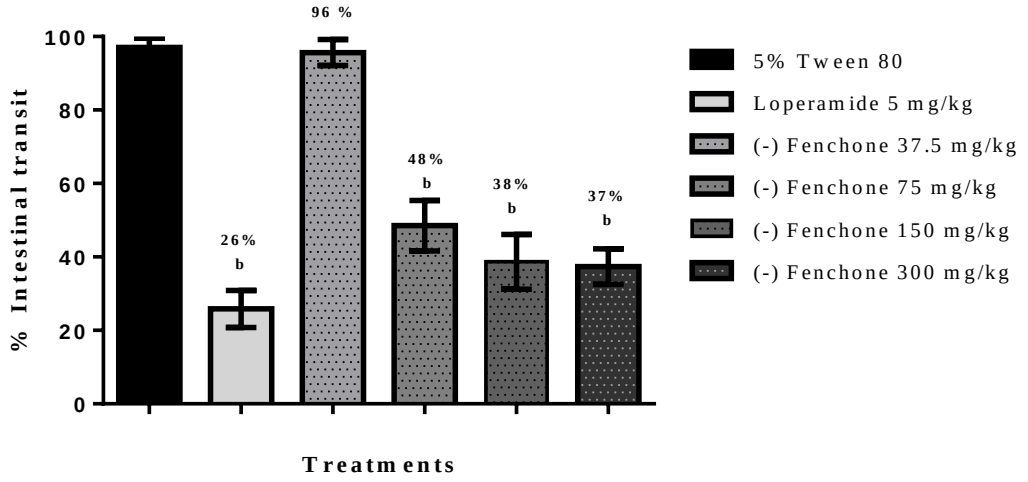
Comment 9. The bibliography should be reduced in number.

Answer: The number of bibliographic references has been reduced, as can be seen in the list of references.

Comment 10. Figures 1 and 2, the columns in the graph should be textured.

Answer: Textures were added to the columns of the graphs in figures 1 and 2.





REVIEWER 3:

- **Thanks for inviting me to review this paper. This is an interesting article and the results are attractive. However, the mechanism should be further proved.**

Answer: The performed experiments have allowed a reliable assessment of the antidiarrheal activity of (-)-fenchone, using classic approaches, which reinforce its reproducibility. The castor oil-induced diarrhea model is considered a universal model; it is the most used method for screening the antidiarrheal activity of new compounds

(Tunaru et al., 2012; Gadacz et al., 1976; Beubler et al., 1979; Racusen et al., 1979). The proposed mechanisms, on the other hand, provide an overview of the different mechanistic pathways (antisecretory and antimotility), which already elucidate or restrict the possible mechanisms of action of the substance under study.

(Tunaru et al., 2012 - https://doi.org/10.1073/pnas.1201627109; Gadacz et al., 1976 - https://doi.org/10.1007/bf01072077; Beubler, 1979 - https://doi.org/10.1111/j.2042-7158.1979.tb13628.x; Racusen, 1979 - https://doi.org/10.1172/JCI109358).

REVIEWER 4:

- **English needs revission.**

Some excerpts of the article were rewritten, and the entire manuscript underwent a new revision of the English language by a native speaker.

REVIEWER 5:

Comment 1. I think the study is interesting. It gathers info about a specific compound in the context of gastrointestinal phenomena using classic approaches. A couple of mechanisms of action were suggested, and this could be further explored. Some alterations are necessary, both in format and content. I would also highlight that there might be a lack of novelty, as indicated by other studies with compounds from the same group that found similar findings. However, it is a novel compound and we could argue this study reinforces reproducibility, which is missing in science these days. It needs some English review by a native speaker.

Answer: This manuscript is the result of an innovative study since we showed for the first time the antidiarrheal and antifungal activity of (-)-fenchone. Classical, standardized, and reproducible methodologies were used, reaffirming the reliability of our data (Tunaru et al., 2012; Gadacz et al., 1976; Beubler et al., 1979; Racusen et al., 1979). Besides, this work also corroborates some studies with other innovative substances that showed similar behavior (Negreiros et al., 2019; Jalilzadeh-Amin; Maham, 2014; Costa et al., 2020). Some excerpts of the article were rewritten, and the entire manuscript underwent a new revision of the English language by a native speaker.

(Tunaru et al., 2012 - https://doi.org/10.1073/pnas.1201627109; Gadacz et al., 1976 - https://doi.org/10.1007/bf01072077; Beubler, 1979 - https://doi.org/10.1111/j.2042-7158.1979.tb13628.x; Racusen, 1979 - https://doi.org/10.1172/JCI109358; Negreiros et al., 2019 - https://doi.org/10.1016/j.biopha.2018.11.131; Jalilzadeh-Amin; Maham, 2014 - https://doi.org/10.3109/13880209.2014.935862; Costa et al., 2020 - https://doi.org/10.1016/j.ejphar.2020.172986).

Comment 2. My specific thoughts: I think the title suggests antidiarrhea effect happens through the antimicrobial activity, and this is misleading. It should also specify antifungal effect instead of antimicrobial in the title. I think the authors should state in the abstract the no effect on bacteria, as these are major causes of diarrhea (more than fungi).

Answer: The title was changed to highlight only the compound'scompound's antifungal activity, to: *"Antifungal activity and antidiarrheal activity via antimotility mechanisms of (-)-fenchone in experimental models"*. The lack of antidiarrheal activity was also emphasized in summary: *This phytoconstituent presents antifungal activity; however, it did not show antibacterial activity"*.

Comment 3. Background is good, with the major info necessary for introducing the study, but I suggest the authors to include epidemiologic data indicating the burden of diarrhea in adults as well. Diarrhea in children from developing countries is mainly related to infections (and great proportion from bacteria) and undernutrition, so a target population for this drug could be adults from high income countries. The authors could highlight this population in the first paragraph.

Answer: The introduction has been changed to include epidemiological data indicating rates of diarrhea in adults, and the leading causes of diarrhea in adults in high-income countries were also highlighted in this section. As can be seen: *"The prevalence of chronic diarrhea is estimated at 1 to 5% of the adult population, and in developed countries (CARRASCO- LABRA et al., 2019)"*. And add: "*In adults, the most common causes of diarrhea include irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), celiac disease, malabsorption of syndromes, and microscopic colitis. The assessment and management of this condition can be a challenge, since the diagnosis is quite broad, especially concerning the differentiation between organic or functional causes that may be involved in its etiology (CARRASCO-LABRA et al., 2019; BURGERS; LINDBERG; BEVIS, 2020)"*.

(Carrasco-Labra et al., 2019 - doi:10.1053/j.gastro.2019.06.014, Burgers; Lindberg; Bevis, 2020 - PMID: 32293842).

Comment 4. I think the bacteria testing should have been focused on the types that are associated with intestinal infections in humans: diarrheogenic E. coli, Salmonella, Shigella, Campylobacter, Yersinia. Staphyloccocus and Pseudomonas are associated with different contexts. This is a limitation.

Answer: Epidemiological studies have shown the importance of *Escherichia coli* as a cause of morbidity and mortality from diarrhea in infants and young children and children under five years of age (KOTLOFF et al., 2013). Estimates show that for the regions of South Asia and Africa, *E. coli* is the cause of 30% of annual episodes of diarrhea among children over five years old and 82% of deaths from global diarrhea among children under five years old (LIU et al., 2012; LAMBERTI et al., 2014). *Escherichia coli* is the most

frequently isolated microorganism, responsible for causing approximately 200 million cases of diarrhea and about 380,000 deaths worldwide (KOTLOFF et al., 2013).

(Kotloff et al., 2013 - doi: 10.1016 / S0140-6736 (13) 60844-2; Liu et al., 2012 - doi: 10.1016 / S0140-6736 (12) 60560-1; Lamberti et al., 2014 - https://doi.org/10.1371/journal.pntd.0002705).

But to explore and expand the possibilities of (-)-fenchone antimicrobial activity against other microorganisms, the strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which although not the main microorganisms causing diarrhea, were also clinically crucial in various contexts. Epidemiological studies have shown that nosocomial infections are responsible for 23,000 deaths per year in Brazil; however, many cases are still not registered due to underreporting in hospitals. Data from the bacterial resistance report that the main microorganisms are causing infections in Brazilian hospitals between the years 2012 and 2015 were *Klebsiella pneumoniae, Acinetobacter spp., Coagulase-negative Staphylococcus, Staphylococcus aureus,* and *Pseudomonas aeruginosa*. Furthermore, they have a high rate of resistance to antimicrobial agents, and in the case of *S.aureus*, approximately 60% of clinical isolates were resistant to oxacillin (ANVISA, 2016).

ANVISA. Agência nacional de vigilância sanitária (ANVISA): Boletim de Segurança do Paciente e Qualidade em Serviços de Saúde nº 14. Brasil, 2016.

Comment 5. Authors must be more objective and focused on the discussion of their findings. If possible, the authors could identify what is needed for having a more complete understanding of why different terpenes show different effects. Is there any pattern? Could the authors suggest any approach to tackle this, such as comparison studies of terpenes?

Answer: The discussion was revised and changed, keeping the focus on discussing the results objectively. Possibly the different effects observed in the different terpenes are related to the chemical and structural differences of these compounds, such as the bonded substituents (functional groups) and the substitution pattern (Griffin, 1999). Thus, it is not yet possible to precisely define a standard behavior profile for the pharmacological effects of terpenes. Therefore, there is still a need for significant research into the chemical and biological properties of these constituents. It would be essential to carry out comparative studies between the terpenes, especially concerning the structure-activity (ex: in-silico), to try to stipulate patterns (Barbieri et al., 2017).

(Griffin, 1999 - doi: 10.1002 / (SICI) 1099-1026 (199909/10); Barbieri et al., 2017 - doi: 10.1016 / j.micres.2016.12.003.).

Comment 6. In the second paragraph, authors speculate whether the compound would show antisecretory effects. I think this is misleading as throughout the text they conclude there'sthere's no antisecretory effects. This sentences houldbe deleted.

Answer: This sentence was deleted, as recommended, to improve understanding of subsequent results.

Comment 7. Info citing studies that relate to a specific finding should be all in a single paragraph if the studies follow the same general logic of results among them. The paragraphs that describe muscle contraction/relaxation mechanisms and muscarinic/adrenergic receptors should just be deleted, in my view (this is the textbook feeling). Specific points giving rationale to a given approach is fair in the context of presenting and discussing your findings, but not like you did.

Answer: The discussion was revised, some paragraphs were rewritten or summarized more clearly and objectively, to improve the understanding of the text. Other items were excluded, such as the physiological mechanisms of muscle contraction/relaxation, as recommended by the reviewer.

For example: *"Sympathetic innervation (via noradrenaline) acts as inhibitory feedback modulating the release of ACh in the myenteric plexus (via presynaptic α2 adrenergic receptors coupled to Gi/Go) and also by its action on receptors present in the intestinal smooth muscle (via post receptors - β2 adrenergic synaptic coupled to Gs). Both actions result in inhibition of peristaltic activity and decreased tone of intestinal smooth muscle, leading to reduced intestinal motility. Therefore, a blockade of pre or post-synaptic receptors can increase intestinal transit. The presynaptic α2 adrenergic receptor antagonist yohimbine or post-synaptic β adrenergic receptor antagonist propranolol were used to induce intestinal motility. It can be observed that in the presence of blockers, the antimotility effect of (-)-fenchone was significantly reduced. Hence, it can be inferred that the adrenergic pathway is related to the antimotility effect of fenchone… These results corroborate with findings of Formiga et al. (2017) with the ethanolic extract of Maytenus erythroxylon Reissek, which has triterpenes in its composition, the administration of the extract associated with yohimbine or propranolol, reduced its antimotility effect, suggesting the participation of adrenergic pathways in their effect".*

Comment 8. The paragraph prior to the one you suggest that the compound influences the peristaltic movements should be shortened and linked to the one you discuss the data. Also, thefollowingparagraph does notcontribute for discussion.

Answer: The discussion was revised, some paragraphs were rewritten or summarized more clearly and objectively, to improve the understanding of the text, as a paragraph about peristaltic movements, being linked to data discussion. The subsequent paragraph was deleted, as recommended by the reviewer.

For example: *"To evaluate if (-)-fenchone influenced gastrointestinal motility, the evaluation of gastric emptying and intestinal transit protocols were assessed. The findings suggested an antimotility activity mediated by (-)-fenchone, since it was efficient in decreasing gastric emptying and intestinal transit. Similar results were found for monoterpene 1.8-cineol showed a reduction in gastric emptying[46]. (-)-fenchone decreased the propulsion of the marker (activated charcoal suspension) through the intestine. It suggests that (-)-fenchone influenced peristaltic movements of the intestine, characterizing an antimotility activity. Silva et al have shown that the monoterpene carvone also reduced the percentage of intestinal transit in this model."*

Comment 9. It would be good to speculate with literature'sliterature's help why this compound does not show antisecretory activities.

Answer: We conducted a literature search on the antisecretory activity of terpenes. Although many terpenes have already shown antisecretory effects in the enteropooling model (α-terpineol (Negreiros et al., 2019), 1,8-cineol (Jalilzadeh-Amin; Maham, 2014) and farnesol (Costa et al., 2020)), some natural products have also been shown to have no significant effects on the accumulation of intraluminal fluid, such as the Combretumlemprosum extract, which did not alter the fluid accumulation in the enteropooling model (Cavalcante et al., 2019). Similar to (-) - fenchone, the antidiarrheal activity of this extract may result mainly from modulation of intestinal motility instead of secretion. Combretumleprosum is rich in terpenes such as arjunolic acid, folic acid, and lupeol. Thus, these phytochemicals can contribute to the antidiarrheal effect of this extract. For a product or substance to have antisecretory activity, it is necessary to negatively modulate the permeability of Cl - channels in enterocytes, including the cystic fibrosis transmembrane conductance regulator (CFTR), in addition to promoting an increase in the intestinal absorption of Na +. Some natural products with antisecretory activity inhibit Cl - channels in experimental models.

(Thiagarajah et al., 2014). (Negreiros et al., 2019 - https://doi.org/10.1016/j.biopha.2018.11.131; Jalilzadeh-Amin; Maham, 2014 - https://doi.org/10.3109/13880209.2014.935862; Costa et al., 2020 - https://doi.org/10.1016/j.ejphar.2020.172986; Cavalcante et al., 2019 - http://dx.doi.org/10.1590/0001-3765201820170932; Thiagarajah et al., 2014 - doi: 10.1016/j.cgh.2013.12.001).

- **The lack of synergism with antifungals and the lack of antisecretory mechanisms put the study drug in disadvantage in comparison with other compounds from the same class? The authors should discuss about the translational potential of the findings.**

The lack of synergism with antifungals and the lack of antisecretory mechanisms of (-) - fenchone does not represent a disadvantage compared to other compounds in the same class. The antimicrobial action of essential oils and their constituents, such as terpenes, is being associated with their high lipophilicity. It facilitates the access to the lipid layer of the bacterial cell membrane and fungal mitochondria, acting to increase the permeability of these structures, resulting in extravasation cell contents and ions, leading to cell lysis (VERGIS et al., 2013; RAUT; KARUPPAYIL, 2014; DAGLI et al., 2015). The lack of synergism may be related to the fact that (-) - fenchone and amphotericin B possibly act by the same mechanism of action, competing for the same binding site, which limits an effect potentiation. Another association study was carried out by Khan, Malik, and Ahmad (2012), in which the monoterpenogeraniol, showed varying degrees of interaction with the standard antifungals fluconazole and amphotericin B, demonstrating synergism for some strains of C. albicans and indifference for others, showing that there is no pattern. Although (-) - fenchone does not show synergy with amphotericin B, this phytoconstituent has a potent antifungal activity and a potential therapeutic agent against fungal infections. Regarding the lack of antisecretory effects, it also does not represent a limitation, since (-) - fenchone showed promising diarrheal effects, mainly involving antimotility effects, which is also an essential mechanism of antidiarrheal action.

(Vergis et al., 2013 - https://doi.org/10.1080/10408398.2012.692127; Raut; Karuppayil, 2014 - https://doi.org/10.1016/j.indcrop.2014.05.055; Dagli et al., 2015 - DOI: 10.4103

/ 2231-0762.165933; Khan; Mailk; Ahmad et al., 2012 - doi: 10.3109 / 13693786.2011.582890).

- **There is a too large paragraph about microbiota, causes and treatment of diarrhea in the discussion. It is repetitive with the introduction.**

Excerpts on the microbiota and surpluses on the causes and treatments of diarrhea were excluded.

Comment 10. Is there any evidence in animals (including in your study) or humans to speculate whether the concentrations found could be safe?

Answer: Data from Sigma Aldrich, supplier of the substance, show that (-) - fenchone has low acute toxicity. LD50 Oral - Rat - female -> 2,000 mg / kg (OECD Test Guideline 423), with no mortality observed at this dose.

(Sigma-Aldrich. Toxicological information Safety Data Sheet (1R)-(−)-Fenchone. https://www.sigmaaldrich.com/MSDS/MSDS/).

Comment 11. It is necessary for the authors to highlight limitations of the study, such as potential other in vivo models of diarrhea that could help to reinforce these data, as well as other simple molecular marker analyses to characterize mechanisms. The lack of data on post-exposure treatment should be cited as well - Why was this not attempted?

Answer: The absence of data on post-exposure treatment is a limitation of the work. However, in this work, acute models are performed. We have the perspective of performing other models of diarrhea in vivo that can help reinforce this data, as well as other analyzes of molecular markers to characterize mechanisms. For example, prostaglandin E2 (PGE2) and cholera toxin induction models, which are agents commonly used to induce diarrhea in animals. Other markers that can be evaluated to characterize the mechanisms of action are the determination of the concentration of chloride ions, interaction with GM1 receptors (of the cholera toxin), calcium channels, and the opioid system.

Comment 12. Also, it needs to address potential cytoxicity of the compound.

Answer: There are not many studies that evaluate the cytotoxic potential of this compound. However, a study by Rolim et al. (2017), determined the cytotoxicity of (-) - fenchone in mouse erythrocytes. In this study, fenchone did not induce a hemolytic effect at a concentration of 3000 μg / mL.

(Rolim et al., 2017 - doi: 10.1186 / s12906-017-1779-z).

Comment 13. In the context novelty, the authors should defend why they think their findings are important for the literature.

Answer: Our findings are necessary for the literature, since, for the first time, we showed the antidiarrheal and antifungal activity of (-) - fenchone. For this, classic, standardized, and reproducible methodologies were used, reinforcing the reliability of the data. Also, this study stands out because it reports on a new molecule with great pharmacological potential, given the current limitations in the treatment of diarrhea and fungal infections.

Step 6: Editorial Office'Office' scomments

The author must revise the manuscript according to the Editorial Office'Office' scommentsandsuggestions, which listed below:

(1) Science Editor: 1 Scientific quality: The manuscript describes a basic study of the antidiarrheal and antimicrobial activity of (-)-fenchone. The topic is within the scope of the WJG. (1) Classification: Grade B, Grade B, Grade B, Grade C and Grade C; (2) SummaryofthePeer-Review Report: The paperispresentingtheresultsof a studyontheanti-diarrhealactivityof a bi-cyclicmonoterpenecompoundfromessentialoils. Thisisaninterestingarticleandtheresults are attractive. However, themechanismshouldbefurtherproved. The experiments are well-designed, and the results are wellpresentedhoweverthemethodsandthe figures are lackingmanyimportantdetails. The questionsraisedbythereviewersshouldbeanswered; and (3) Format: There are 3 tablesand 6 figures. A total of 79 references are cited, including 23 referencespublished in thelast 3 years. Thereis 1 self-citation. 2 Languageevaluation: Classification: Grade B, Grade B, Grade B, Grade B and Grade B. 3 Academicnormsandrules: The authorsprovidedtheBiostatistics Review Certificate, thesignedConflict-of-InterestDisclosureFormand Copyright LicenseAgreement, theInstitutional Animal Careand Use CommitteeApprovalForm, andthe ARRIVE guideline.

Comment 1: The authorsneedtoprovide original Institutional Review Board Approval Form.

Answer: The certificate of approval of the institutional review board will be sent in PDF format.

No academic misconduct was found in the Cross-Check detection and Bing search. 4 Supplementary comments: This is an unsolicited manuscript. CAPES and CNPq supported the study. The topic has not previously been published in the WJG. The corresponding author has not published articles in the BPG.

Comment 2: 5 Issuesraised: (1) I found the authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s):

Answer: The study was not funded by CAPES and CNPq agencies, so we do not have the approved grant application form (s). The study was funded by the

institution (UFPB). The funding agencies CAPES and CNPQ supported scholarships for students.

Comment 3: (2) I foundtheauthorsdidnotprovidethe original figures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs orarrowsortextportionscanbereprocessedbythe editor;

Answer: The figures were saved in the PowerPoint format and will be sent in a separate file.

Comment 4: (3) I foundtheauthorsdidnotwritethe ""articlehighlight"" section. Pleasewritethe ""articlehighlights"" sectionattheendofthemaintext;

Answer: The "article highlights" section was written and included at the end of the main text.

Comment 4: (4) pleasedon’t include any *, #, †, §, ‡, ¥, @….in yourmanuscript; Please use superscriptnumbers for illustration; and for statisticalsignificance, please use superscriptletters. Statisticalsignificanceisexpressed as aP< 0.05, bP< 0.01 (P > 0.05 usually does notneedtobedenoted). Ifthere are other series of P values, cP< 0.05 anddP< 0.01 are used, and a third series of P valuesisexpressed as eP< 0.05 andfP< 0.01.

Answer: Statistical significance was expressed using superscript letters, with a description in the figure's label.

Point-to-Point response for the second-round review

WORLD JOURNAL OF GASTROENTEROLOGY - MANUSCRIPT

NO: 56930

REVIEWER 1:

Comment 1: Abstract: Aim: To investigate...

Answer: The text was modified to "To investigate antidiarrheal activity related to gastrointestinal motility, intestinal secretion and antimicrobial activity."

Comment 2: Abstract: Conclusion: could be without" hence, it is possible to infer that" ... my opinion is to write clear what was demonstrated.

Answer: All text has been modified to “The antidiarrheal effect of (-)-fenchone in this study involves antimotility effect and not involve antisecretory mechanisms. (-)-Fenchone presents antifungal activity; however, it did not show antibacterial activity.”

Comment 3: Material and methods: on the paragraph about gastric emptying, please rephrase the sentence about " one zero-time control... "

Answer: The text has been change to " After 1h the animals received the 10 mL/kg phenol red-colored marker (0.05% phenol red-colored in 1.5% carboxymethylcellulose - thickening agent). To the non-treated control group (the zero-time control group), the phenol red-colored marker was administrated and the mice were immediately euthanized. The treated groups received the same marker and were euthanized 30 min after administration."

Comment 4: Material and methods: also do not use abs if not explained before using in the formula.

Answer: The text has been modified to “% Gastric emptying = (100 - mean absorbance of sample)/Mean absorbance of zero-time control group× 100.”

Comment 5: Results: in the paragraph about the intestinal transit: please correct - results of this study showed that..., or the study proved that...

Answer: The text has been change to "Results of this study showed that the distance travelled by a marker (activated charcoal) in terms of percentage of the total length of the intestine was 97% in the negative control group. Treatment with loperamide (5 mg/kg, p.o.) and (-)-fenchone at doses of 75, 150 and 300 significantly reduced ($p < 0.001$) the percentage of intestinal transit to 26, 48, 38 and 37%, respectively, when compared to the control group."

Comment 6: Results: Association assay, please check the first phrase... both above and below?

Answer: All text has been modified to “Tests demonstrated that (-)-fenchone maintained the MIC of 32 µg/mL and amphotericin B the MIC of 02 µg / mL for the strains tested, maintaining the MIC at equal values both individually and in an association."