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Research Article

Evaluation of anti-diarrhoeal activity of L-citrulline in mice

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L-citrulline, anti-diarrhoeal activity, Loperamide, castor oil.

ABSTRACT

Background: L-citrulline is a naturally occurring physiological non-essential amino acid that plays an important role in the metabolism and regulation of nitric oxide. Nitric oxide is important for physiologic processes of gastrointestinal tract, like motility and absorption. L citrulline is majorly synthesized in the small intestine and considered safe for consumption. However, there is paucity of literature on its anti-diarrhoeal effects. Hence, this study investigated the anti-diarrhoeal activity of L-citrulline in mice. **Methods:** Anti-diarrhoeal and anti-enteropooling effects of L-citrulline were evaluated by inducing diarrhoea and enteropooling with castor oil. The effect of L-citrulline on normal intestinal motility was also evaluated using charcoal maker. L-citrulline (300 and 600 mg/kg) was administered to test groups, Loperamide (5 mg/kg) was administered to the positive control groups and Normal saline (2ml/kg) was administered to negative control groups. All administrations were via oral route. The results were analyzed using one-way Analysis of variance and Dunnett's post-hoc test. **Results:** The control groups in all parameters evaluated showed typical diarrhoeal signs. Diarrhoea protections of 93.33% and 55.49% were observed at 300 and 600 mg/kg of L-Citrulline, respectively. L-Citrulline inhibited fluid accumulation by 35.88% and 28.27% at 300 and 600 mg/kg, respectively. The mean percentage distance travelled by the charcoal maker was inhibited by 13.76% and 2.62% at 300 and 600 mg/kg, of L-citrulline, respectively. The observed antidiarrhoeal effects of L-citrulline could be attributed to its ability to inhibit both intestinal motility and fluid accumulation in the mice. **Conclusion:** This study has shown that L-citrulline possess some anti-diarrhoeal potentials. However, there is need for further anti-diarrhoeal studies using other models and lower graded doses of L-Citrulline to further elucidate L-Citrulline anti-diarrhoeal mechanism of action.

INTRODUCTION

Diarrhoea is one of the most important dysfunctions in the intestinal tract that occur due to increase in bowel motility. It is the frequent passage of loose stool in such a way that it is abnormal for the individual (WHO, 2017; Osonwa *et al*., 2016). Imbalances in the absorptive and secretory processes of the intestines leads to diarrhoea (Chitme *et al*., 2004; Udedi *et al*., 2013). Infectious agents (virus, bacteria, parasites and fungi), toxins from plants and animals and substances that are poorly absorbed by the intestine all cause diarrhoea. In diarrhoeal stools, large amounts of sodium, potassium together with water are lost. This leads to dehydration, hypovolemia, cardiovascular collapse and death if left untreated. Diarrhoeal disease is a leading cause of death in developing countries (Bairagi *et al*., 2014; Misra *et al*., 2014), it kills about 525,000 children less than 5 years of age annually (WHO, 2017).

L-citrulline is a naturally occurring physiological nonessential amino acid that derive its name from watermelon *Citrullus vulgaris* from which it was first isolated. It plays a very important role in the metabolism and regulation of nitric oxide. L-citrulline is generally recognized as safe for oral consumption and

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the Lethal Dose (LD_{50}) of L-citrulline has been reported to be greater than 5,000 mg/kg body weight in both rats and mice (Fagron, 2012; Kyowa, 2013). Also, there is growing interest in the use of L-citrulline as a nitric oxide related dietary Supplement (Suzuki *et al*., 2016). It has various beneficial effects, such as ameliorating arterial stiffness (cardiovascular function), improving erectile function, memory, oxygen uptake kinetics, immuno-stimulation, protein metabolism and high-intensity exercise (ergogenic effect) performance through upregulation of nitric oxide synthesis (Davis *et al*., 2011). Despite the large body of evidence of L-citrulline beneficial effects, there is paucity of literature on its anti-diarrhoeal effects.

Also, the management of diarrhoea at present involves the use of anti-motility, anti-secretory, anti-fungi and anti-bacteria agents and/or oral rehydration therapy (ORT) (Otimenyin and Uzochukwu, 2010) with zinc (Alam *et al*., 2017). There is need for further search for more effective agents. Therefore, this study aimed at investigating the anti-diarrhoeal activity of L-citrulline in mice.

MATERIALS AND METHODS

Drugs, Chemicals, and Materials

L-Citrulline Pure Powder (NOW FOODS, 395 S. Glen Ellyn Rd. Bloomingdale, IL 60108, U.S.A), Castor oil (Bell Sons and Co. Ltd, Southport PR9 9AL, England), Loperamide (Jiangsu Ruinian Qianjin Pharm. Co., Ltd.). Normal saline (0.9% w/v sodium chloride solution), Activated charcoal and Gum acacia used for the study were purchased commercially and were of Analytical grade.

Experimental Animals

Sixty (60) mice (Swiss albino) were used in this study. They were of either sex and weighed between 18-24 grams. They were obtained and kept in standard cages at the Department of Pharmacology and Therapeutics animal house, Ahmadu Bello University, Zaria. Their cages were cleaned on regular bases and they were fed on commercial feeds with tap water ad libitum. Ethical clearance was obtained from Ahmadu Bello University committee on animal use and care (Approval No. ABUCAUC/2018/033) and the experiments were carried out in accordance with the rules governing the use of laboratory animals (Garber *et al*., 2011).

Experimental Design (Animal Grouping and Dosing)

The sixty (60) mice were divided into three (3) study groups each consisting of twenty mice. Each study group was used for anti-diarrhoeal, enteropooling and normal intestinal transit studies respectively. The study groups were further divided into four sub groups of five (5) animals each. Each sub-group received the following treatment orally on the day of the experiment after been fasted for 18 hours: group I were given normal saline 2 ml/kg to serve as negative control, group II were given standard drug Loperamide 5 mg/kg to serve as positive control, group III and IV were given L-Citrulline 300 mg/kg and 600 mg/kg respectively.

Determination of Anti-diarrhoeal Activity Castor Oil Induced Diarrhoea

The methods described by Awouters *et al*., (1978) and Diurno *et al*., (1996) were adopted for this study. The mice were grouped and treated as described above. Diarrhoea was induced in each mouse 1 hour after treatment by oral administration of 0.2 ml of castor oil. Each mouse after receiving castor oil was immediately placed in a separate cage whose floor was lined with white paper. After each hour, the paper was changed until the fourth hour. During observational period the total number of wet faeces was recorded. The average of the recorded results was calculated and then use to calculate percentage protection against diarrhoea using the formula below:

$$\% \text{ Protection} = \frac{\text{Average No. of WFC - Average No. of WFT}}{\text{Average No. of WFC}} \times 100$$

WFC = Wet feaces in Control Group, WFT = Wet Feaces in Test Group/Standard Drug

Castor Oil Induced Enteropooling

The methods described by Robert *et al*., (1976) and DiCarlo *et al*., (1994) were adopted for this study to determine Intraluminal fluid accumulation. The mice were grouped and treated as described above in the experimental design. Enteropooling was induced in each mouse 1 hour after treatment with oral administration of 0.2ml of castor oil. After an hour of castor oil administration each mouse was sacrificed by cervical dislocation and the abdomen was immediately cut open. The small intestine from the pylorus to the caecum was ligated and the whole length was dissected

and carefully removed. Thereafter, the intestine was weighed, and the contents were collected by milking into a graduated tube to measure the volume. The empty intestine was also reweighed and the difference between the two weights were calculated. The mean of the difference in weight and the mean volume of the small intestinal content were calculated. The percentage inhibition of volume of the small intestinal content was determined using the formula below:

$$\% \text{ Inhibition} = \frac{\text{MVSIC of Control Group} - \text{MVSIC of Test Group/SD}}{\text{MVSIC of Control Group}} \times 100$$

MVSIC = Mean Volume of Small Intestinal Content, SD= Standard Drug

Intestinal Motility Test

The methods described by Than *et al*., (1989) and Bako *et al*., (2013) with some modification were adopted for this study. The mice were grouped and treated as described above in the experimental design. After 30 minutes of treatment 0.3 ml of charcoal maker (made up of 5% aqueous suspension of activated charcoal deactivated with 3% Gum acacia) was administered orally to each mouse. After keeping the mice that received the charcoal maker for 1 hour, they were sacrificed by cervical dislocation and the abdomen of each mouse was immediately opened. The intestine was dissected out and carefully stretched. The total length of the intestine as well as the distanced covered by the charcoal maker from the pylorus was recorded. The mean distance covered by the maker was calculated and used to determine the percentage inhibition of normal motility using the formula below:

$$\% \text{ Inhibition} = \frac{Dc - Dt}{Dc} \times 100$$

Dc =Mean distance travelled by the charcoal maker in control group,
Dt = Mean distance travelled by the charcoal maker in other groups apart from the control

Statistical Analysis

Data obtained are expressed as Mean ± Standard Error of Mean (M ± S.E.M). The result was analyzed using one-way Analysis of variance (ANOVA) followed by Dunnett's post-hoc test to compare level of significance between other groups and the control. SPSS version 20 was used for the analysis and P values < 0.05 was considered as significant.

RESULTS

Effects on Castor oil-induced diarrhoea

Standard drug Loperamide and L-Citrulline 300 mg/kg significantly reduced the total number of wet feaces when compared to the control group. Loperamide was observed to show higher percentage protection of 100% against diarrhoea while L-Citrulline 300 mg/kg showed 93.33% protection against diarrhoea. On the contrary, L-Citrulline 600 mg/kg which showed a percentage protection of 55.49% did not significantly reduce the total number of wet feaces when compared to the control group. (Table 1).

Table 1: Effect of L-Citrulline on castor oil-induced diarrhoea

Groups	Treatment	Dose	Total No. of diarrhoeal feaces	% Protection of diarrhoea
I	Normal Saline	2ml/kg	5.60 ± 1.08	0.00%
II	Loperamide	5mg/kg	0.00 ± 0.00*	100.00%
III	L-Citrulline	300mg/kg	0.60 ± 0.60*	93.33%
IV	L-Citrulline	600mg/kg	2.60 ± 1.44	55.49%

Values are expressed as mean ± SEM. (n = 5).*P < 0.05, Dunnett's test as compared to negative control (Normal saline).

Effect on castor oil- induced enteropooling

Standard drug Loperamide, L-Citrulline 300 mg/kg and 600 mg/kg significantly reduced the weight and volume of small intestinal content when compared to the control group. Maximum percentage inhibition of volume of intestinal content by L-Citrulline was observed at 300 mg/kg being 35.88%. The percentage inhibition by L-Citrulline at 300 mg/kg and 600 mg/kg are lower when compared to Loperamide which showed higher percentage inhibition of volume of intestinal content of 40.05% (Table 2).

Table 2: Effect of L-Citrulline on castor oil-induced enteropooling

Groups	Treatment	Dose	MWSIC (g)	MVSIC (ml)	% Inhibition by using MVSIC
I	Normal Saline	2ml/kg	0.54 ± 0.02	0.55 ± 0.01	0.00%
II	Loperamide	5mg/kg	0.34 ± 0.02*	0.33 ± 0.02*	40.05%
III	L-Citrulline	300mg/kg	0.38 ± 0.04*	0.35 ± 0.02*	35.88%
IV	L-Citrulline	600mg/kg	0.40 ± 0.03*	0.39 ± 0.01*	28.27%

Values are expressed as mean ± SEM. (n = 5).*P 0.05, Dunnett's test as compared to negative control (Normal saline).
MWSIC = Mean Weight of Small Intestinal Content, MVSIC = Mean Volume of Small Intestinal Content.

Effect on intestinal motility

Standard drug Loperamide and L-Citrulline 300 mg/kg inhibited normal intestinal motility by significantly reducing the distance travelled by the charcoal meal when compared to the control group. L-Citrulline 600 mg/kg on the contrary did not significantly reduce the distance travelled by the charcoal meal when compared to the control group. Loperamide was observed to show the highest percentage inhibition (35.22%) of intestinal motility (Table 3).

Table 3: Effect of L-Citrulline on normal intestinal motility in mice

Groups	Treatment	Dose	Total Distance of Intestine (cm)	Distance Travelled by Charcoal Meal (cm)	% Inhibition of Motility
I	Normal Saline	2ml/kg	47.02 ± 0.67	47.02 ± 0.67	0.00%
II	Loperamide	5mg/kg	46.94 ± 0.92	30.40 ± 0.90*	35.22%
III	L-Citrulline	300mg/kg	47.14 ± 0.86	40.50 ± 1.17*	13.76%
IV	L-Citrulline	600mg/kg	46.62 ± 1.26	45.78 ± 0.97	2.62%

Values are expressed as mean ± SEM. (n = 5).*P < 0.05, Dunnett's test as compared to negative control (Normal saline).

DISCUSSION

These study was carried out to investigate the antidiarrhoeal activity of L-Citrulline in Mice. The experimental model used in this study were castor oil induced diarrhoea and enteropooling model. Lipase enzymes in the intestine act on castor oil to cause the release of its active metabolite ricinoleic acid (Kulkami and Pandit, 2005). Ricinoleic acid act on the small intestine to cause increase in motility and intraluminal fluid by altering the normal peristaltic activity and changing the permeability of sodium and chlorine in the intestinal mucosa. Also, ricinoleic acid stimulates the secretion of prostaglandins (particularly those of the E series) which causes diarrhoea in animals and humans (Tunaru *et al*., 2012; Rahman *et al*., 2013). The effect of L-Citrulline observed in the castor oil induced diarrhoea model was not dose dependent as its only L-Citrulline 300 mg/kg that significantly reduced the mean number of wet feaces, while L-Citrulline 600 mg/kg did not. However, in the castor oil induced enteropooling model, L-Citrulline at both 300 mg/kg and 600 mg/kg significantly inhibited intraluminal fluid accumulation. Also, L-Citrulline 300 mg/kg was observed to show more percentage protection against diarrhoea and maximal percentage inhibition of intraluminal fluid accumulation. Loperamide whose antidiarrhoeal effect has been well documented showed maximal percentage protection against diarrhoea (Faure, 2013; Rahman *et al*., 2013). The observed effect of L-Citrulline could be attributed to nitric oxide since L-Citrulline has been documented to increases the level of nitric oxide in the body (Jabłecka *et al*., 2012; Suzuki *et al*., 2016). Nitric oxide causes vasodilation and increased blood flow to viscera's (McKinley-Barnard *et al*., 2015; Suzuki *et al*., 2016). Increase in blood flow to the gastrointestinal tract will enhance absorption of electrolytes and water thereby reducing intraluminal fluid as well as the number of wet feaces produced (Yacob *et al*., 2016). These findings are in line with the studies of Moinard *et al*., 2007 who reported that L-Citrulline taken acutely at a dose up to 15 g does not cause diarrhoea, however in this present studies L-Citrulline 600mg/kg did not significantly protect the mice against diarrhoea in the castor oil induce diarrhoea model. In the normal intestinal motility studies, L-Citrulline 300mg/kg significantly inhibited normal intestinal motility, by reducing the mean distance travelled by the charcoal maker, while L-Citrulline 600 mg/kg did not. The maximal inhibition of motility was observed with Loperamide which act by inhibiting intestinal motility (Tadesse *et al*., 2017). The observed effect of L-Citrulline can therefore be attributed to its ability to

decrease intestinal motility by reducing the distance moved by the charcoal maker. The observed antimotility effect of L-Citrulline is in line with the findings of Ruiz and Tejerina, 1998, who reported that L-Citrulline may have actions complementary to those of nitric oxide in the control of smooth muscle relaxation.

In general, increase in fluid absorption and/or slowing of intestinal motility may account for the observed anti-diarrhoeal effect of L-Citrulline. This may explain why L-Citrulline could protect the mice against diarrhoea induced by castor oil. However, the decline in percentage protection of diarrhoea, percentage inhibition of intestinal fluid accumulation and percentage inhibition of normal intestinal transit with increasing dose of L-Citrulline may be attributed to poor absorption of higher dose of L-Citrulline in the intestine. As it has been documented that there is little or no evidence for an increase or decrease uptake of LCitrulline by enterocytes under low or high dose (Romero *et al.,* 2006).

CONCLUSION

The present study shows L-Citrulline inhibited motility and secretion in the intestine and this contributed to its anti-diarrhoeal effects. However, L-Citrulline at 300 mg/kg showed more significant effect. The antidiarrhoeal effect observed could be attributed to L-Citrulline acting alone and or by increasing the level of nitric oxide. Therefore, further studies using other models of diarrhoeal with lower graded doses of L-Citrulline as well as studies on the effect of L-Citrulline on nitric oxide expressed in the small intestinal smooth muscle, can be carried out in other to further elucidate L-Citrulline mechanism of action.

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