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Anti-nociceptive and anti-inflammatory activities of the methanol extract of *Waltheria americana* Linn. leaf in experimental animals

Idowu Olumorin Owemidu[1], Mujeedat Adebukola Olubori[1], Oluwaseun Samuel Faborode[1,2], Oluwatobi Stephen Oloyede[1], Samuel Adetunji Onasanwo[1]

[1]Neurosciences and Oral Physiology Unit, Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Nigeria

[2]Department of Human Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Bingham University, Karu, Nigeria.

ABSTRACT

Background: *Waltheria americana* has been used in African folklore for the treatment of minor and complicated ailments like pain, fever, and rheumatism.
Aim: To investigate the analgesic and anti-inflammatory potentials of *Waltheria americana* and its possible mechanism of action in Swiss mice and Wistar rats, respectively.
Materials and Methods: Methanol extract of *Waltheria americana* leaf was evaluated for anti-nociceptive activities (using tail flick, formalin-induced paw licking, and acetic acid-induced writhing tests in mice) and possible mechanisms of action using atropine (5 mg/kg ip), prazosin (1 mg/kg po), glibenclamide (8 mg/kg po), propranolol (40 mg/kg po), naloxone (2 mg/kg ip), nifedipine (10 mg/kg po) using acetic acid, and tail-flick models of analgesia. The anti-inflammatory effect was evaluated in carrageenan-induced paw edema and cotton pellet-induced granuloma tests.
Results: Acute toxicity revealed no mortality in the mice up to a dose of 2,000 mg/kg. The phytochemistry revealed the presence of alkaloids, flavonoids, saponins, and terpenoids. The extract (100 or 200 mg/kg) produced inhibition ($p < 0.05$) both in the formalin-induced paw licking and acetic acid-induced writhing tests. In the thermal test, the extract (200 mg/kg) significantly increased the withdrawal latency. There was a reversal of the analgesic effect of the extract with the cholinergic and opioidergic blockers that significantly produced changes in the analgesic effects of the extract. The extract produced dose-related inhibition of cotton pellet-induced granuloma in rats and carrageenan-induced paw edema in rats.
Conclusion: The extract produced anti-inflammatory potentials and anti-nociception through mechanisms that may depend on the cholinergic and opioidergic system.

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Anti-nociception; *Waltheria americana*; nifedipine; prazosin; atropine; anti-inflammatory

Introduction

Medicinal plants are relied upon for the prevention and treatment of many health problems among rural populations globally, which improves the quality of life. The cost and access to modern drugs among the rural populations of tropical Africa made a large proportion of rural people to depend on traditional herbal drugs [1].

Waltheria americana L. belongs to the family *Sterculiaceae*, also known as velvetleaf, marshmallow, monkey bush, boater bush, etc [2]. It is found throughout the tropics and warmer subtropics. *Waltheria americana* grows on disturbed areas, roadside weed, old pastures, cotton fields, rock crevices on top of plains, inundated savannas, riverbanks, forests borders or slopes, impoverished soils, on limestone, or basalt rock outcrops [3]. In traditional medicine, *Waltheria americana* is used for the treatment of minor ailments (e.g., sore throat, cough) and complicated ailments (e.g., inflammation, asthma). Its roots were used in the treatment of wounds [4], leprosy [5], fever and pain [6],

Contact Samuel Adetunji Onasanwo ✉ samphil2002@yahoo.com or sa.onasanwo@gmail.ui.edu.ng Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Nigeria.

rheumatism [4], night blindness, gum and teeth disease, diarrhea, and dysentery [7]. Preliminary phytochemistry has revealed the presence of alkaloids [8], terpenoids and saponins [9], and flavonoids [10].

Several flavonoids were isolated from *Waltheria indica* as reviewed by Zongo and co-workers [11]. These include (-)-epicatechin, tiliroside [12], quercetin [12,13], and kaempferol [13,14]. A rare acylated flavonol glycoside—Kaempferol-3-O-β-D-(6"-E-p-coumaryl)-glucopyranoside was isolated from the whole plant [13].

Flavonoid derivatives from *Waltheria indica* were evaluated *in vitro* and showed that tiliroside, (-)-epicatechin, and quercetin induced a dose-dependent inhibition of the production of the inflammatory mediators, including nitric oxide, tumor necrosis factor-α, and interleukin-12 [12].

It is noteworthy that most of the drugs used presently for the management of pain possess more toxic effects [15]. On the contrary, many medicines of plant origin are economical in the treatments of different ailments. However, there are few reports on the analgesic and anti-inflammatory activities of *Waltheria americana* leaf. Therefore, the purpose of this study is to evaluate the analgesic and anti-inflammatory effects of the methanol extract of *Waltheria americana* (MEWA) leaf to support the folkloric claim, and the possible mechanisms of action using different experimental models of analgesia and inflammation.

Materials and Methods

Animals

A total of 135 male Swiss mice (22–30 g) were used for the acute toxicity and analgesic studies and 40 Wistar rats (150–200 g) were used for the inflammatory study. They were acclimatized for 2 weeks, kept under standard laboratory conditions, and fed on rodent cubes (Ladokun Feeds, Ibadan, Nigeria). All experimental procedures on rodents were conducted in accordance with established protocols under the guidelines of the Principle of Laboratory Animal Care (National Institute of Health publication No. 85-23) [16] and ethical guidelines for investigation of experimental pain in conscious animals by Zimmerman [17].

Collection and extraction of Waltheria americana *leaf*

Waltheria americana leaves were obtained around College of Agriculture, Kabba, Kogi state, and authenticated by Mr. Odewo S. A. and Mr. Adeniji K. A. of the Herbarium, Forestry Research Institute of Nigeria, Ibadan, Nigeria where the plant was kept with a voucher number FHI: 111064. The leaves were air-dried at room temperature and finely powdered with a blender. Four hundred grams of the pulverized plant was macerated in methanol for 72 hours. After the extraction, the extract was sieved and filtered. The filtrate was concentrated in the oven at 40°C [18]. The dried extracts were stored at 4°C until needed. Appropriate dose dilutions were made with distilled water.

Acute oral toxicity test

Twenty-five adult male Swiss mice (20–30 g) were divided into five groups. Each group consists of five mice [19]. The mice fasted for 24 hours. Groups 1–5 received 50, 100, 200, 1,000, and 2,000 of MEWA leaf. The mice were observed continuously for 2 hours daily for behavioral and autonomic profiles, and for any sign of toxicity or mortality, up to a period of 14 days.

Phytochemical screening

The extract was screened to detect the presence of some phytochemicals according to the methods described by Sofowora [20].

Drugs and chemicals

The following drugs and chemicals were used prazosin (alpha-adrenergic blocker), atropine (a non-selective muscarinic receptor antagonist), indomethacin (Cyclooxygenase Cox inhibitor), propranolol (Beta-adrenergic blocker), nifedipine (L-type voltage-gated calcium channel blocker), acetic acid, ketamine (anesthesia), formaldehyde, methanol, naloxone (a non-selective opioid receptor antagonist), glibenclamide (an Adenosine triphosphate (ATP)-sensitive K^+ channel inhibitor). They were of high analytical value.

Analgesic screening

Chemical and thermal models were used in this study. All tests were conducted under the ethical guidelines of the International Association for the Study of Pain [21].

Acetic acid-induced writhing test in mice

The acetic acid-induced abdominal constriction test was carried out with minor modifications [22]. In order to induce pain in mouse peritoneal cavity, 0.6% of acetic acid (10 ml/kg) was injected

intraperitoneally 60 minutes after the administration of MEWA leaf (50, 100, and 200 mg/kg po). The number of abdominal constrictions was counted cumulatively between 5 and 15 minutes after acetic acid administration. Anti-nociception of MEWA leaf was indicated by the reduction in the mean of the number of abdominal constrictions in the test groups compared to the control group. Indomethacin (10 mg/kg po) was used as reference drug while the control group received distilled water (10 ml/kg po).

Formalin-induced paw licking test in mice

The procedures used in the formalin-induced paw licking test was similar to [23] with minor modification. Animals were pre-treated with MEWA leaf (50, 100, and 200 mg/kg po) 60 minutes before the formalin injection. Control animals received only distilled water (10 ml/kg po) while (indomethacin 10 mg/kg po) was used as the reference drug. After 60 minutes, the intraplantar area of the right hind paw of the mice was injected with 20 µl of 2.5% of formalin. The animals were then immediately placed individually in a transparent observation chamber and the time the animal spent licking the injected paw was recorded for 30 minutes following formalin injection [first 5 minutes after formalin injection (early phase) and 15–30 minutes after formalin injection (late phase)].

Tail-flick latency assay in mice

Ugo Basile tail-flick 37360 model maintained at 55°C was used and the time taken for the animal to withdraw its tail was recorded as withdrawal latency [24]. The animals were grouped as above, the control received (10 ml/kg po) distilled water, the reference group received (10 mg/kg po) indomethacin, and the other groups received (50, 100, and 200 mg/kg po) of the extract.

Mechanism of anti-nociception of the methanol extract of Waltheria americana *leaf*

The possible mechanism of action of *Waltheria americana* leaf was investigated using the acetic acid-induced abdominal writhing test and tail-flick test. The animals were pre-treated with *Waltheria americana*, distilled water (10 ml/kg) 60 minutes before acetic acid (0.6%, ip), and before the tail-flick test. Alpha-adrenergic blockers (prazosin, 1 mg/kg po); beta-adrenergic blocker (propranolol, 40 mg/kg po); muscarinic cholinergic blocker (atropine, 5mg/kg ip); L-type voltage-gated calcium channel blocker (nifedipine, 10 mg/kg po); non-selective opioidergic receptor blocker (naloxone, 2 mg/kg ip); and ATP-sensitive K^+ channel blocker (glibenclamide, 8 mg/kg po) were given before the extract, against the probable analgesic potential of MEWA leaf.

Anti-inflammatory effects of methanol extract of Waltheria americana *leaf in carrageenan-induced paw edema model of inflammation*

Carrageenan-induced rat paw edema was done by the method of Winter and a co-worker with a slight modification [25]. Inflammation was induced by the injection of 0.1 ml of freshly prepared carrageenan (1%) aqueous suspension in normal saline underneath the plantar tissue of the right hind paw of rats. The different groups of rats were administered with MEWA (100 and 200 mg/kg po) and indomethacin (10 mg/kg po). The control group received vehicle (distilled water, 10 ml/kg po). One-hour after treatment, paw edema was induced by the injection of edematogenic agent carrageenan. The paw volume was measured by a Plethysmometer (Ugo Basile model: 37140). The measures were determined at 0 hour (V_o: before edematogenic agent injection) and 1, 2, 3, 4, and 5-hour intervals later (V_t). The difference between V_t and V_o was taken as the change in paw volume (edema value).

Anti-inflammatory effects of methanol extract of Waltheria americana *leaf in cotton pellet-induced granuloma model of inflammation*

The effect of MEWA leaf on the chronic phases of inflammation was assessed in the cotton pellet-induced granuloma rat model [26]. Sterilized cotton pellets weighing 30 mg was implanted subcutaneously through a small ventral incision in anesthetized rats. The different groups of rats were treated with MEWA leaf (100 and 200 mg/kg po) and indomethacin (10 mg/kg po) once daily for seven consecutive days from the day of cotton pellet insertion. The control group received vehicle (distilled water, 10 ml/kg po). On the eighth day, the animals were sacrificed and the cotton pellets were removed; dried at 60°C for 24 hours, and their mass was determined. Mean weight of the granuloma tissue formed around each pellet was obtained and percentage inhibition was expressed as compared with the control group.

Statistical analysis

Data were presented as mean ± Standard Error of Mean (SEM). Comparisons between groups were made using the one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test, 95%

confidence level, and at $p < 0.05$ was considered statistically significant.

Results

Acute toxicity

There was no mortality recorded in any of the animals treated with varying doses 50, 100, 200, 1,000, and 2,000 mg/kg of the MEWA leaf. During the observation period of 14 days, all the treated animals were found to be healthy and normal without any apparent symptoms of adverse effects.

Preliminary qualitative phytochemical analysis of methanol extract of Waltheria americana *leaf*

The yield was 2.4%, and the phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, and terpenoids in the MEWA leaf as shown in Table 1.

Effects of methanol extract of Waltheria americana *(MEWA) leaf on acetic acid-induced writhing test in mice*

Figure 1 shows that the extract (100 and 200 mg/kg) produced a significant ($p < 0.05$) reduction in writhing movements caused by acetic acid. The reference drug indomethacin 10 mg/kg also produced a significant reduction in writhing movements caused by acetic acid. The extract dose (200 mg/kg) has the highest inhibition which is comparable to the reference drug indomethacin (10 mg/kg).

Effects of methanol extract of Waltheria americana *leaf on tail-flick latency in mice*

Figure 2 shows the effects of graded doses of the MEWA leaf on tail-flick latency(s). The extract (200 mg/kg) tested showed significant ($p < 0.05$) increase in the tail-flick latency(s), the extract (50 and 100 mg/kg) were not significant compared with the control (distilled water 10 ml/kg). The potency of the 200 mg/kg of the extract was comparable to the reference group (indomethacin, 10 mg/kg).

Table 1. Preliminary qualitative phytochemical analysis of MEWA leaf.

Phytochemicals	Methanol extract
Alkaloids	+
Flavonoids	+
Saponins	+
Tannins	–
Anthraquinones	–
Terpenoids	+
Cardiac glycosides	–

+ = means present; – = means not present.

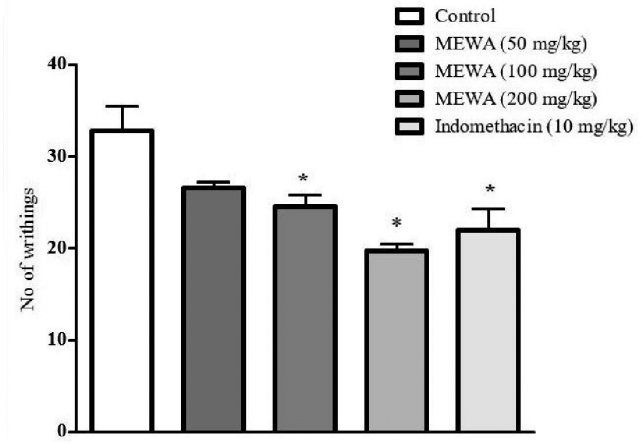

Figure 1. Effects of graded doses of MEWA leaf on acetic acid-induced writhing in mice. Data represent means ± SEM of five mice. Comparisons were made using one-way ANOVA, followed by Dunnett's post-hoc test. *p < 0.05 compared to control.

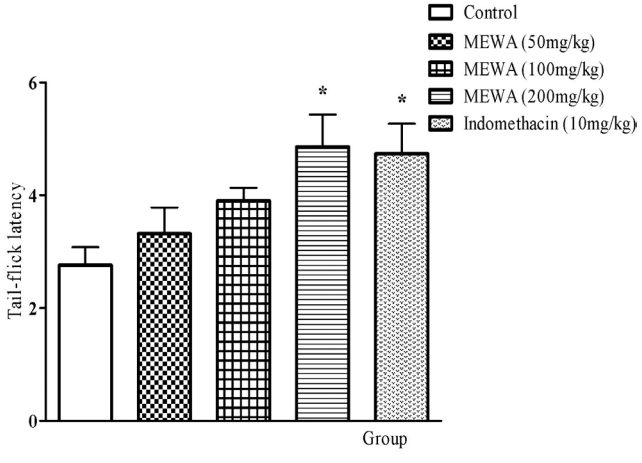

Figure 2. Effects of graded doses of MEWA leaf on tail-flick latencies in mice. Data represent means ± SEM of five mice. Comparisons were made using one-way ANOVA, followed by Dunnett's post-hoc test. *$p < 0.05$ compared to control is significant.

Effects of Waltheria americana *on formalin-induced paw licking test in mice*

From Figure 3, extract doses (200 and 100 mg/kg) significantly ($p < 0.05$) and dose-dependently decreased the early and late phases. All the extract doses (50, 100, and 200 mg/kg) and the reference drug indomethacin (10 mg/kg) seem to exert an effect on the early phase than on the late phase.

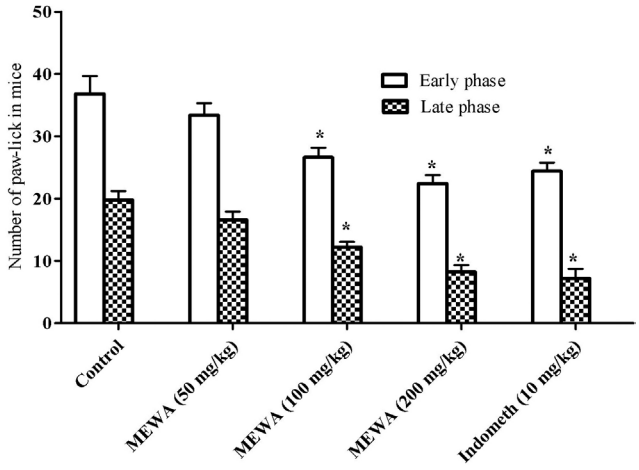

Figure 3. Effects of graded doses of MEWA leaf on formalin-induced paw-lick in mice. Data represent means ± SEM of five mice. Comparisons were made using one-way ANOVA, followed by Dunnett's post-hoc test. *$p < 0.05$ compared to control is significant.

Effects of pre-treatment with adrenergic, cholinergic, L-Type voltage-gated calcium channel, opioidergic receptor, and ATP sensitive K⁺ channel blocker in the tail flick and acetic acid-induced writhing in mice

From table 2, the pretreatment with propranolol, prazosin, nifedipine, and glibenclamide did not significantly affect the tail-flick latency and acetic acid-induced writhing in mice. But pre-treatment with atropine and naloxone significantly reversed the effect of the extract as compared with the control (200 mg/kg *Waltheria americana* leaf).

Anti-inflammatory effects of methanol extract of Waltheria americana *leaf in cotton pellet-induced granuloma model of inflammation*

The results of the anti-inflammatory activity of MEWA leaf in cotton pellet-induced granuloma is shown in Table 3. The extracts 100 and 200 mg/kg, and the reference drug (indomethacin 10 mg/kg) showed a significant decrease in granuloma formation as compared to the control group. The percentage change produced by the extract 100 mg/kg, 200 mg/kg, and indomethacin 10 mg/kg are 39.13%, 56.52%, and 52.17%, respectively.

Table 2. Effects of pretreatment with adrenergic, muscarinic cholinergic receptors, L-Type voltage-gated calcium channel blocker, Opioidergic receptor blocker, and ATP sensitive K⁺ channel blocker followed by 200 mg/kg *Waltheria americana* leaf on tail flick test and acetic acid-induced writhing in mice.

Group	Latency	Numbers of writhing
Waltheria americana (control)	4.18 ± 0.14	15.00 ± 1.71
Prazosin + extract	3.94 ± 0.44	16.00 ± 0.68
Propranolol + extract	4.40 ± 0.48	14.00 ± 0.93
Atropine + extract	2.66 ± 0.17*	26.00 ± 2.00*
Nifedipine + extract	5.20 ± 0.51	16.00 ± 1.6
Naloxone + extract	2.70 ± 0.20*	29.40 ± 1.81*
Glibenclamide + extract	4.04 ± 0.43	18.20 ± 1.46

Data represent means ± SEM (n = 5 mice. Comparisons were made using one-way ANOVA, followed by Dunnett's post-hoc test. *$p < 0.05$ compared to control.

Anti-inflammatory effects of methanol extract of Waltheria americana *leaf in carrageenan-induced paw edema in rats*

The results of carrageenan-induced inflammation in Table 4 showed a decrease in paw size of all the treated groups compared with that of the control group. At 2, 4, and 5 hours post-carrageenan administration, the paws of the extract (100 and 200 mg/kg) with the indomethacin-treated animals decreased significantly ($p < 0.05$) compared with the control group. After 1 hour of carrageenan

Table 3. Anti-inflammatory effects of *Waltheria americana* leaf in cotton pellet-induced granuloma in rats.

Treatment groups	Dose (mg/kg)	Pellet weight (g/100g b.w)	% change
Control	10	0.023 ± 0.001	-
MEWA	100	0.014 ± 0.002*	39.13
MEWA	200	0.010 ± 0.001*	56.52
Indomethacin	10	0.011 ± 0.001*	52.17

Data represent means ± SEM of five mice. Comparisons were made using one-way ANOVA, followed by Dunnett's post-hoc test. *$p < 0.05$ compared to control is significant.

Table 4. Anti-inflammatory effects of MEWA leaf in carrageenan-induced paw edema in rats.

Groups	Change in paw volume (ml)					
	After 0 hour	After 1 hour	After 2 hours	After 3 hours	After 4 hours	After 5 hours
Control	1.30 ± 0.15	2.53 ± 0.15	3.08 ± 0.10	3.30 ± 0.10	1.42 ± 0.10	1.11 ± 0.10
Indomethacin (10 mg/kg)	0.69 ± 0.10*	1.190 ± 0.15*	2.26 ± 0.06*	2.60 ± 0.05*	0.67 ± 0.05*	0.43 ± 0.05*
MEWA (200 mg/kg)	0.74 ± 0.18*	1.86 ± 0.22*	2.26 ± 0.21*	2.55 ± 0.28*	0.55 ± 0.12*	0.40 ± 0.11*
MEWA (100 mg/kg)	0.70 ± 0.11*	1.96 ± 0.21	2.16 ± 0.21*	2.71 ± 0.12	0. 60 ± 0.11*	0.42 ± 0.12*

Data represent means ± S.E.M of five mice. Comparisons were made using one-way ANOVA, followed by Dunnett's post-hoc test. *$p < 0.05$ compared to control is significant.

administration, the reference drug (indomethacin, 10 mg/kg) was the only group that showed a significant decrease in paw size. Meanwhile, at 3 hours post-carrageenan administration, the extract (100 mg/kg) did not show a significant decrease in paw size compared to the control group.

Discussion

This study was carried out to investigate the anti-nociceptive and anti-inflammatory effects of the MEWA leaf. Phytochemical analysis of *Waltheria americana* leaf revealed the presence of alkaloids, flavonoids, saponins, and terpenoids. The alkaloids, the largest single class of secondary plant substances, have been reported by researchers to exhibit a remarkable range of pharmacological activities. These include analgesic, anti-inflammatory, and anti-cancer activities [27–29], and this may be responsible for the analgesic and anti-inflammatory effect of the extract. Chemical and thermal models were employed to check the analgesic potentials of *Waltheria americana* while the cotton pellet-induced granuloma and the carrageenan-induced paw edema were used for the anti-inflammatory study.

The acetic acid-induced writhing is normally used to test for new analgesic agents [30]. The intraperitoneal injection of acetic acid usually leads to the release of inflammatory mediators like substance P, bradykinin, serotonin, histamine, and prostaglandins which stimulate the primary afferent nociceptors to send a nerve impulse to dorsal horn neurons of the Central nervous system (CNS) leading to abdominal constrictions [31]. There was an inhibition of this abdominal constrictions in animals treated with the extract as compared with the control, suggesting that the extract possibly prevents the release of inflammatory mediators. This was also supported by comparing the potentials of the cyclooxygenase (COX) inhibitor, indomethacin with 200 mg/kg of the extract which were comparable. This test suggests that the extract may act by the inhibition of COX and other inflammatory mediators. Further tests were employed as muscle relaxants could cause data misinterpretation in the acetic acid-induced writhing test [32].

The formalin test has two phases; the early phase is the neurogenic phase as a result of direct stimulation of sensory nerve fibers while the late phase referred to as the inflammatory phase is caused by the release of chemical mediators including histamine, bradykinin, serotonin, and substance P [33]. This study showed that both phases were significantly reduced by the extract as compared with the control which was similar to the observation of Onasanwo and Elegbe [34]. Conventionally, centrally acting drugs are well known to inhibit both early and late phases significantly [35]. Therefore, the inhibition of both the early and late phases in this test together with the reduction of abdominal constrictions in the acetic acid-induced writhing test showed that the extract of *Waltheria americana* leaf acts centrally and peripherally.

The tail-flick test is based on a phasic stimulus of high intensity; the nociceptive experience is short-lasting and they are thus, models of acute pain [35]. As investigated in this study, the MEWA leaf produced increased withdrawal latency, and this result shows that the extract has anti-nociceptive activity in acute pain. Thermally-induced nociceptive responses in mice could be inhibited by narcotic agents as centrally acting analgesics [36]. This result implies that the analgesic effect of the extract is centrally mediated. Thus, the increased nociceptive threshold in this test together with the reduction of nociception in the formalin-induced paw licking test (early and late phases) also supports the evidence of centrally mediated anti-nociceptive activity of MEWA leaf.

Chronic inflammation leads to the development of proliferative cells. These cells are either spread or in granuloma form, in this study the anti-inflammatory activity of the MEWA leaf has been established using the cotton pellet granuloma model. The dry weight of the pellet correlates with the amount of granuloma tissue [27]. The extract showed a significant decrease in the weight of the pellet. This reflects the efficacy of the extract in chronic inflammatory conditions.

Carrageenan has been used as an agent for inducing experimental inflammation, which is a screening tool for agents with the anti-inflammatory property. Carrageenan-induced rat paw edema is suitable *in vivo* model to predict the value of anti-inflammatory agents which act by inhibiting the mediators of acute inflammation [37]. The early phase of carrageenan-induced paw edema is due to the release of serotonin, histamine, and similar substances while the late phase leads to the activation of kinin-like substances such as prostaglandins, proteases, and lysosomes [38]. The MEWA leaf inhibits both early and late phase, which shows that the effect may be by preventing the release of inflammatory mediators, mostly the COX products.

More so, arachidonate COX inhibitors due to its COX-dependent mechanism control this test effectively. Thus, the extract of *Waltheria americana* leaf may possess arachidonate COX inhibitory property. In the investigation of the mechanism of the anti-nociceptive activity of MEWA leaf, administration of adrenoceptors blockers (prazosin and propranolol), L-type voltage-gated calcium channel blocker (nifedipine) and ATP-sensitive K^+ channel did not affect the anti-nociception of *Waltheria americana* leaf except for the pretreatment with muscarinic cholinergic blocker (atropine) and non-selective opioidergic blocker (naloxone) which significantly abolished the anti-nociception of *Waltheria americana* leaf, showing that the analgesic activity of MEWA leaf is non-adrenergic dependent, does not involve L-type voltage-gated calcium channel and ATP sensitive K^+ channel. But the cholinergic and opioidergic systems might be involved in the mechanism of action of the extract of *Waltheria americana* leaf.

Conclusion

In conclusion, this study clearly revealed the analgesic and anti-inflammatory activity of the MEWA leaf, and showed that the mechanism of action of anti-nociception may not be due to the adrenergic system, ATP sensitive K^+ channel and does not involve L-type voltage-gated calcium channel; but the cholinergic and opioidergic systems may be involved in the anti-nociceptive activity of the MEWA leaf.

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Kolaviron modulates intestinal motility and secretion in experimentally altered gut functions of Wistar rats

Olugbenga Adeola Odukanmi, Adeola Temitope Salami, Oyenike Lola Morakinyo, Oluwole Emmanuel Yelotan, Samuel Babafemi Olaleye

Laboratory for Gastrointestinal Secretion and Inflammation Research, Department of Physiology, University of Ibadan, Ibadan, Nigeria

ABSTRACT

Aim: The effect of kolaviron, a complex of *Garcinia kola* (GK) naturally rich in bioflavonoid, was investigated on intestinal motility and secretion in altered gut functions of rats.

Methods: Four experiments were carried out using male Wistar rats (189.1 ± 3.5 g). The first was for intestinal transit with charcoal meal, and rats were grouped into four (*n* = 5/group in all experiments): control (DMSO); atropine (5 mg/kg), 100 mg/kg (KV100) kolaviron group, and 200 mg/kg (KV200) kolaviron group, respectively. Experiments 2 and 3 were to assess diarrhea and enteropooling, respectively; the animals were grouped into six: negative control (DMSO), positive control (castor oil), atropine (5 mg/kg), loperamide (3 mg/kg), KV100 and KV200, respectively. Experiment 4 was to determine colonic motility and it consists of five groups: negative control (DMSO), positive control (Serotonin, 5 mg/kg, ip), atropine (5 mg/kg), KV100, and KV200 in turn.

Results: Kolaviron significantly decreases intestinal transit in a similar way to the atropine group, KV200 (27.3%), and KV100 (25.7%) compared with control. The onset of diarrhea was prolonged significantly while episodes of loose stool and purging index decreased significantly with loperamide (111.0 minutes, 0.4 ± 0.2, 0.1) and KV200 (128.8 minutes, 2.6 ± 0.7, 2.0) compared with control (52.6 minutes, 6.6 ± 1.0, 15.6), respectively. Kolaviron significantly reduced luminal fluid in KV100 (1.04 ± 0.17 ml) and KV200 (0.62 ± 0.21 ml) compared with control (1.70 ± 0.18 ml). Colonic motility was delayed in KV200 (182 ±18.7 seconds) compared with control (139 ± 8.72 seconds).

Conclusion: Kolaviron exhibits potent anti-motility and anti-secretory activities on destabilized gut homeostasis and could be the major compound of GK responsible for previously reported antidiarrheal effect.

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Introduction

The contact time for food and nutrients with the mucosa epithelium is reduced whenever the gut is quick. However, a lot of nutrients are lost consequent to the resultant diarrhea. Diarrhea is a common but daring symptom that could result in loss of massive fluid and electrolytes. It is the second leading cause of death in children under 5 years old worldwide [1–3]. Diarrhea spans throughout both developed and developing worlds; resulting from miscellaneous conditions with the highest mortality recorded in sub-Saharan Africa [4]. The annual incidence of diarrhea is about 1.7 billion cases globally and approximately a million mortality yearly [3]. However, it is not exceptionally a disease of the children as adults do have it and could die from it if not properly managed. Certain conditions predispose adults to diarrhea such as poor hygiene (infection), chronic diseases (HIV/AIDS and inflammatory bowel disease), and poor nutrition (high carbohydrate diet).

Diarrhea is also defined as an alteration in the normal bowel movement and involves the passage of watery or loose stool more than normal for an individual [5]. It could be associated with an increase in luminal fluid secreted, the rate of bowel movements and sounds, diarrheic stools, and at times abdominal discomfort. It is associated with

Contact Olugbenga Adeola Odukanmi ✉ odukanmi@yahoo.com Laboratory for Gastrointestinal Secretion and Inflammation Research, Department of Physiology, University of Ibadan, Ibadan, Nigeria.

Table 1. Animal groupings according to the different experiments.

S/N	Experiments	Groups					
		nControl	pControl	Atropine	Loperamide	KV100	KV200
1	Intestinal transit test	-	✓	✓	-	✓	✓
2	Diarrhea test	✓	✓	✓	✓	✓	✓
3	Enteropooling test	✓	✓	✓	✓	✓	✓
4	Colonic motility test	✓	✓	✓	-	✓	✓

n = 5 in each group.

poorly absorbed nutrients and fluid while water and electrolytes losses are enormous [6–9].

Numerous plants from all continents have traditionally been used in the treatment of diarrhea disease [10]. Recently, it was reported that about 80% of the World's population comprising of majorly people living in the developing world now depend almost completely on traditional medicine, using plants as local treatments [11–14]. Reasons alluded to this trend are economic viability, accessibility, and ancestral experience [15]. *Garcinia kola* (GK) Heckel (family *Guttiferae*) is an herb grown in Nigeria and some other parts of the West African forest with a characteristic astringent, bitter, and resinous taste like raw coffee beans. The seed of "bitter kola" (as it is commonly called) is highly used in traditional hospitality and chewed as an alternative to true kola nuts (*Cola nitida* and *Cola accuminata*). GK is a highly valued ingredient in African ethnomedicine because of its varied uses which are social and medicinal; thus making the plant an essential ingredient in folk medicine [16–18]. Chemical investigations of GK seeds have shown that they contain a complex mixture of phenolic compounds, including Garcinia Biflavonoid (GB)-type biflavonoids, xanthones, triterpenes, and benzophenones, as well as cycloartenol and its 24-methylene derivative [19]. The biflavanones are the most dominant in the *Garcinia* species [20].

Several flavonoids have been linked to the inhibition of intestinal motility, especially during *in vivo* experiments [21,22]. GK is reportedly rich in bioflavonoids [23,24] and was earlier reported to have anti-intestinal motility property [24]. Likewise, kolaviron, a rich bioflavonone compound of GK has been described previously for its usefulness as a potent analgesic and anti-inflammatory properties on mice following acetic acid induced abdominal constrictions [25]. The gastro-protective function of kolaviron following chemical and mechanical injuries via antioxidant generation and suppression of reactive oxygen radicals was clearly elucidated by Olaleye and Farombi [26] and Odukanmi et al. [27], respectively. Further to the beneficial roles of kolaviron, is the well clarified modulatory effect it has on intestinal glucose uptake in normal rats [28]. However, there is a dearth of information on its activity on gut motility and secretion. In this study, kolaviron was investigated for its probable influence on altered intestinal motility and luminal fluid accumulation.

Materials and Methods

Animal husbandry, grouping, and ethics

Adult male Wistar rats (189.1 ± 3.5 g) were obtained from Central Animal House, College of Medicine, University of Ibadan. The rats were used for the intestinal transit, diarrhea test, enteropooling, and colonic motility studies. They were housed under standard conditions and fed with standard rodents' pellets and water. Four experiments were carried out using male Wistar rats (189.1 ± 3.5 g). The first was for intestinal transit with charcoal meal, rats were grouped into four (*n* = 5/group in all experiment): control Dimethyl Sulfoxide (DMSO); atropine (5 mg/kg), 100 mg/kg Kolaviron (KV100) kolaviron group, and 200 mg/kg (KV200) kolaviron group, respectively. Experiments 2 and 3 were to assess diarrhea and enteropooling, respectively; the animals were grouped into six: negative control (DMSO), positive control (castor oil), atropine (5 mg/kg), loperamide (3 mg/kg), KV100, and KV200, respectively. Experiment 4 was to determine colonic motility and it consists of five groups: negative control (DMSO), positive control (Serotonin, 5 mg/kg, ip), atropine (5 mg/kg), KV100, and KV200 in turn (Table 1). The experiments were carried out in line with the guidelines for animal experimentation in the Gastrointestinal Secretions and Inflammation Unit of the Department of Physiology, University of Ibadan. It conformed to the Guidelines of the National Institute of Health—*Guide for the Care and Use of Laboratory Animals [29].*

Chemicals

Atropine, loperamide, serotonin, activated charcoal, acacia gum, and castor oil were obtained from Santa Cruz®, Germany.

Extraction of kolaviron

Seeds of GK were obtained locally in Ibadan, Nigeria and certified by a Botanist in the University of Ibadan. A voucher specimen is available in the herbarium of the university. Kolaviron was then isolated according to Iwu et al. [30] as modified by Farombi et al. [31]. Briefly, the seeds were sliced, air-dried, and grinded into powder. The powdered form was defatted by n-hexane in Soxhlet extractor for 24 hours. This gave dried marc which was re-packed and further extracted using methanol. Chloroform was then used to fractionate the concentrated methanolic extract to give a golden yellow solid substance known as kolaviron.

Determination of small intestinal transit

The method described by Teke et al. [32] was adopted for the evaluation of the effect of KV on small intestinal transit in rats. Twenty healthy Wistar rats were grouped into four (n = 5): control, atropine, KV100 group, and KV200 group. All animals were fasted for 18 hours prior to the administration of charcoal meal. The KV groups received an oral single dose and atropine group received 5 mg/kg of atropine sulphate intraperitoneally, 1 hour prior to the administration of activated charcoal. The negative control group received an equivalent amount of DMSO (vehicle) 1 hour prior to administration of charcoal meal through an oral gavage. The charcoal meal (1 ml) (10% charcoal and 5% acacia gum suspended in distilled water and made up to 100 ml of solution) was administered by an oral gavage to all the groups. The animals were sacrificed 30 minutes after the charcoal meal was given by ketamine overdose (100 mg/kg) followed by cervical dislocation. The small intestine was removed carefully and lengths of intestine, as well as the leading end of the charcoal meal were measured. The percentage of distance covered by the charcoal was calculated and used to determine the percentage inhibition.

$$\%\,\text{intestinal transit} = \left(\frac{\text{distance traveled by charcoal meal}}{\text{length of the small intestine}}\right) \times 100$$

$$\%\,\text{Inhibition of transit} = \frac{\text{length of the small intestine} - \text{distance traveled by charcoal}}{\text{length of small intestine}} \times 100$$

Assessment of diarrhea

Rats were deprived of food for 18 hours but had free access to water prior to the experiment. Five groups of Wistar rats (n = 5) were used for the experiment: control, KV groups (KV100 and KV200), atropine and loperamide groups all received 2 ml of castor oil. Kolaviron (100 and 200 mg/kg, po), atropine (5 mg/kg, ip), and loperamide (3 mg/kg, po) were given 1 hour prior to castor oil administration. Each rat was subsequently placed separately in a plastic cage lined at the bottom with a transparent foil sheet. Observations were made for 4 hours, to determine the onset of diarrhea, total number of both wet and dry feces, percentages of rats with diarrhea, and those protected from diarrhea according to the method described by Bajad et al. [33] with slight modifications.

Assessment of castor oil-induced enteropooling

The method described by Chitme et al. [34] was used for the castor oil-induced enteropooling study with slight modifications. Rats were deprived of food but allowed access to water for 18 hours prior to the experiment. Five groups of animals (n = 5) were used: the control received DMSO, KV groups (100 and 200 mg/kg, po), atropine group (5 mg/kg, ip), and loperamide group (3 mg/kg, po). After 1 hour of administration of treatments, 2 ml of castor oil was administered orally to all the groups. All the rats were anaesthetized with an overdose of ketamine (100 mg/kg bw) after 1 hour of administration of castor oil. The abdomen was carefully opened and the intestine was ligated from the two ends, the pyloric and the ileo-cecum junctions. The small intestine was gently isolated from the animals and its contents collected into a clean crucible and measured in a 10 ml measuring cylinder. Percentage inhibition of luminal content was expressed as shown below:

$$\%\,\text{Inhibition of Intestinal Content} = \left\{\frac{\text{Control Mean} - \text{Test Mean}}{\text{Control Mean}}\right\} \times 100\%$$

Determination of colonic motility time

The animals were fasted for 24 hours but given free access to water prior to the experiment. Five groups of rats (n = 5) were used for the experiment. The control was given the equivalent volume of DMSO which was the vehicle; KV groups received 100 and 200 mg/kg kolaviron while atropine group got 5 mg/kg of the drug. All administrations were given

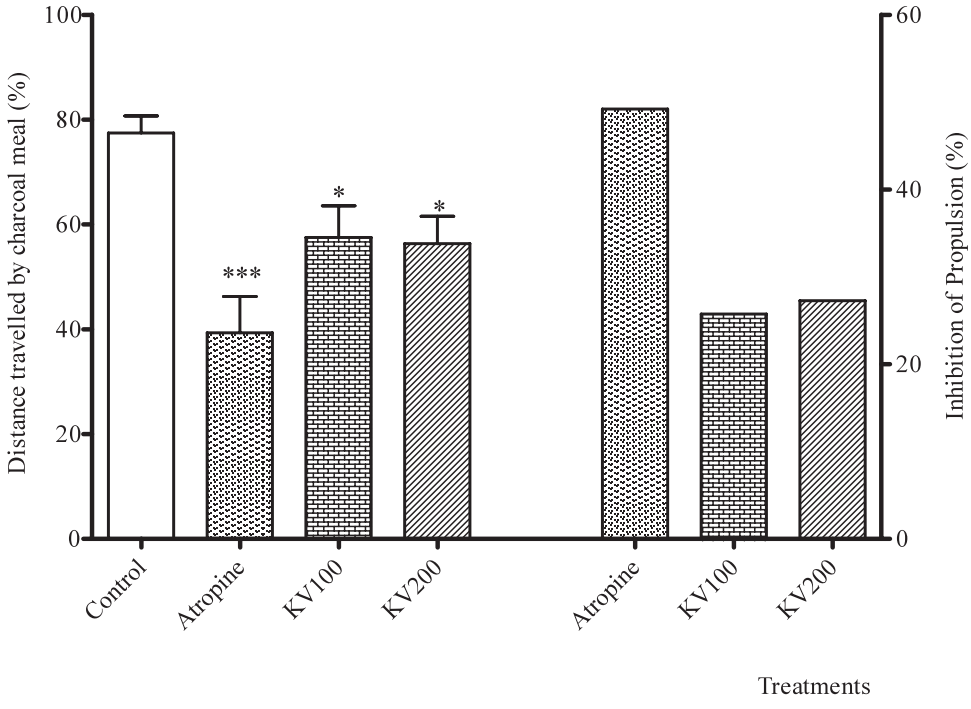

Figure 1. Intestinal transit and inhibition percentages following treatments with kolaviron. ***$p < 0.001$ and *$p < 0.05$ significant when compared with control.

by a gavage except atropine that was administered intraperitoneally. Serotonin (5 mg/kg, ip) was given to all groups 1 hour after the administration of the test substances to promote gut motility. Thereafter, four 3 mm gold colored plastic beads were introduced to the distal 2 cm end of the colon from the anal opening with the aid of a lubricated glass rod. Each rat was then placed in a plastic cage lined at the bottom with a transparent foil sheet. The time taken to expel the first bead was obtained in seconds from the time of insertion. The experiment was performed according to the method described by Coates et al. [35].

Data analysis

Results were expressed as Mean ± Standard Error of Mean (SEM) and analyzed using one-way analysis of variance (ANOVA), Newman-Keuls *Post hoc* test was adopted using GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA), $p < 0.05$ was considered significant.

Results

Effect of kolaviron on charcoal meal transit in the small intestine of rats

The transit of charcoal meal within the small intestine reduced significantly in all the test groups, KV100 mg/kg (54 ± 8.0%), KV200 mg/kg (52 ± 8.2%), $p < 0.05$ and atropine (38 ± 8.4%) at p<0.001 compared with control (78 ± 2.0%), ((Fig. 1). This means that compared with the control group the atropine group and KV groups (100 and 200 mg/kg) had a delayed charcoal meal transit with the percentage inhibition of 49.2%, 25.7%, and 27.3%, respectively.

Effect of kolaviron on castor oil induced diarrhea in rats

Table 2 shows that pre-treatment of the rats with kolaviron delayed the onset of diarrhea significantly in KV100 (115.6 ± 13.3 minutes) and KV200 (128.8 ± 8.1 minutes) compared with the control (52.6 ± 5.4 minutes). The loperamide group produced the largest delay in the onset of diarrhea with the time of 111.3 ± 9.6 minutes. None of the rats treated with kolaviron were protected from diarrhea and the same was observed for the control. The frequency of defecation reduced significantly in the KV100 (5.4 ± 0.9), KV200 (2.6 ± 0.7), and loperamide (0.4 ± 0.4) compared with control (8.2 ± 1.1).

Effect of kolaviron on castor oil induced enteropooling in the small intestine of rats

Table 3 shows that kolaviron exerted a significant fluid secretion inhibitory effect against castor oil-induced activity in all the test groups; KV100 (0.62 ± 0.21 mL), KV200 (1.04 ± 0.17 mL), atropine

Table 2. Effect of Kolaviron on castor oil induced diarrhea.

Treatment groups	Onset of diarrhea (minute)	Rats with diarrhea (%)	% of rats protected	Total number of feces	Number of wet feces	Number of dry feces	Purging index
nControl	-	0	100	1.2 ± 0.7**	0.0 ± 0.0***	1.2 ± 0.7^{ns}	-
pControl	52.6 ± 5.4	100	0	8.2 ± 1.1	6.6 ± 1.0	1.6 ± 0.7	15.6
Atropine$_{(ip)}$	202.5 ± 11.3***	40	60	0.8 ± 0.2***	0.6 ± 0.4***	0.2 ± 0.2^{ns}	0.2
Loperamide	111.3 ± 9.6 ***	20	80	0.4 ± 0.4***	0.4 ± 0.4***	0.1 ± 0.1^{ns}	0.1
KV100	115.6 ± 13.3**	100	0	5.4 ± 0.9*	5.4 ± 0.9^{ns}	0.6 ± 0.2^{ns}	4.7
KV200	128.8 ± 8.1**	100	0	2.6 ± 0.7**	2.6 ± 0.7**	0.0 ± 0.0^{ns}	2.0

One-way ANOVA + Newman-Keuls *Post hoc* test, $n = 5$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to pControl. ns = no significance, $p > 0.05$ compared with pControl.

Table 3. Luminal fluid contents and inhibition of luminal fluid in treated groups.

Treatment groups	Dosage (po)	Luminal fluid contents (ml) (Mean ± SEM)	Inhibition of luminal fluid (%)
nControl	2 ml/rat	0.26 ± 0.07***	84.7
pControl (Castor oil)	2 ml/rat	1.70 ± 0.18	-
Loperamide	3 mg/kg	1.60 ± 0.21^{ns}	5.9
Atropine (ip)	0.6 mg/kg	0.82 ± 0.22*	51.8
KV100	100 mg/kg	0.62 ± 0.21**	63.5
KV200	200 mg/kg	1.04 ± 0.17*	38.8

One-way ANOVA + Newman-Keuls *Post hoc* test, $n = 5$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns = not significant compared to pControl.

(0.82 ± 0.22 mL) compared to that of the positive control group of rats (1.70 ± 0.18 ml). The percentage luminal fluid inhibition compared to control were 63.5% (KV100), 38.8% (KV200), 5.9% (Loperamide), and 51.8% (Atropine).

Effect of kolaviron on serotonin induced alteration in colonic motility of rats

Figure 2 shows that treatment with KV100 (181.5 ± 26.2 seconds), KV200 (115 ± 10.3 seconds), and atropine group (186.5 ± 14.1 seconds) delayed colonic motility significantly when compared to the positive control group (95.4 ± 5.6 seconds).

Discussion

This study was conducted in order to assess the effect of kolaviron, a rich natural biflavonoid compound of GK [36] on gut motility and secretion during experimentally altered gut states. The expectation was to investigate and relate the anti-diarrhea effect of GK earlier reported [24] in the relation of kolaviron. Alteration of gut motility was substantively demonstrated using chemically-induced simulations such as activated charcoal for intestinal transit, castor oil for diarrhea and enteropooling tests, and serotonin for colonic motility.

Decrease in the small intestinal transit reported for kolaviron groups after priming with activated charcoal is comparable to those of gossypin, epicatechin, and hydroxyethyl rutosides—these are natural flavonoids that were reported to have inhibited intestinal transit significantly in mice [21]. In a similar study conducted by Di Carlo et al [22], the authors reported inhibition in intestinal transit with certain flavonoids such as apigenin, flavone, kaempferol, morin, myricetin, naringin, and rutin in mice and rats. These authors in the same study further revealed that some flavonoids such as hesperitin, catechin, and phloridzin had no effect on intestinal transit at the reported dose of 200 mg/kg. It is clear that flavonoids aid in the inhibition of intestinal transit. Conversely, this may not be true for all flavonoids as evidenced by the report of Di Carlo et al. [22]. The reason for this may lie within the structure of individual flavonoid and their respective receptors. Kolaviron inhibited intestinal transit in a similar version as atropine sulphate and flavonoid component might be responsible for this effect.

Castor oil was used to induce diarrhea and has been reported to be effective in animal and human due to its ricinoleic acid component which is the active metabolite [37–39]. The ricinoleic acid causes disruption of the local intestinal milieu by triggering irritation, inflammation, and elevating prostaglandin production thereby promoting an increase in fluid accumulation within the intestine [40] and could also present with toxicity to the intestinal absorptive cells [41]. In the current study, diarrhea was established in most groups but in terms of the time of onset, the frequency of wet feces, and purging index, kolaviron reduced the variables examined

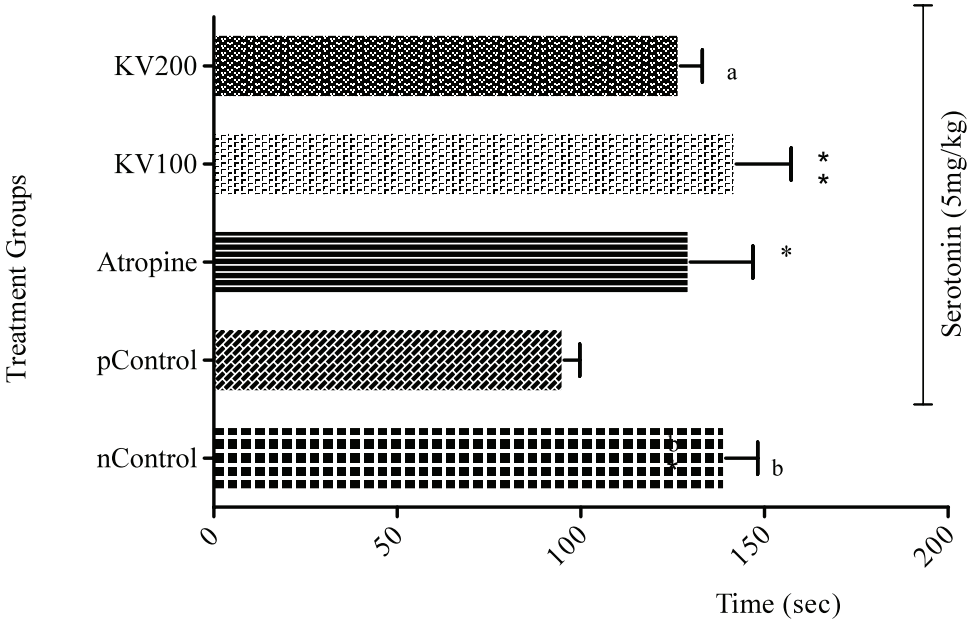

Figure 2. Effect of kolaviron on serotonin-induced altered gastrointestinal motility in rats. ** = colonic motility time increased significantly at $p < 0.01$ compared with pControl group. * = colonic motility time increased significantly at $p < 0.05$ compared with pControl. a = colonic motility time increased significantly at $p < 0.05$ compared with pControl group. b = colonic motility time increased significantly at $p < 0.05$ compared with pControl.

for diarrhea index in correlation with the standard drug loperamide, an opiod receptor agonist. This decrease in purging index reported could be due to the bioflavonoid component of kolaviron. The findings on the role played by flavonoids in resolving diarrhea is well documented [21,22].

In the clinical setting, diarrhea could present in the diverse form, from dysfunctional bowel which might results from malabsorption, undue intestinal secretion of fluid and electrolytes, as well as a swift intestinal movement [42]. Similarly, certain drugs that promote intestinal motility and diarrhea frequency could also affect intraluminal secretions [43]. Apparently, from this study, kolaviron reduced intraluminal fluid secretion by inhibiting castor oil induced excessive fluid secretion. This further buttresses the earlier statement that it could act by inhibiting motility as well as blocking the effect of castor oil from enhancing fluid pooled within the luminal space and so emerging as a better agent than loperamide (the standard drug) in this instance. The reason might be due to its dual role as anti-motility and anti-secretory. On the other hand, a high rate of intestinal absorption might result in decrease intraluminal fluid pooled and together with increased transit time [44]. This is further proven by the small volume of intestinal contents recorded for kolaviron in this study.

Research on serotonin (5-HT) and gut motility has been richly studied for over five decades. Majority of the 5-HT receptors in the body resides within the gut wall, where it is present in different divisions of mucosal epithelial cells (enterochromaffin cells) and neurons (descending interneurons) [45]. Several research propose that 5-HT in the normal and dysfunctional gut is essential for motility and certain medications interacting with 5-HT receptors, particularly 5-HT_3 and 5-HT_4 receptors, have been used clinically to treat gut motility disorders [44].

Accordingly, in this study, serotonin induced increase in colonic motility was repressed by kolaviron. Kolaviron actively prolonged colonic motility time in this study and in relation to the effect of atropine sulphate, a muscarinic antagonist. The prolonged colonic motility time means that colon motility was reduced thereby resulting in retaining the beads for a longer time than the control. This coupled with the earlier submission further substantiated kolaviron's anti-diarrheal and anti-secretory efforts.

In conclusion, it was apparent that kolaviron reduces motility in both upper and lower gut as

well as reduce intraluminal fluid pooled in the small intestine. These were achieved in related mode to the muscarinic receptor blocker and opioid receptor agonist used for the experiments. Therefore, kolaviron exhibits potent anti-motility and anti-secretory effect on destabilized gut homeostasis in this study and could be the major compound of GK responsible for the antidiarrheal effect earlier reported.

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ORIGINAL RESEARCH

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Effects of extract of *Urtica dioica* L. (stinging nettle) on the immune response of rats with severe malnutrition

Sara Beatriz Herrera[1,2], Leonor Rodriguez[2], María del Carmen García[3], José Luis Flores[2], Rodolfo Velasco[2]

[1]Posgrado en Biología Experimental, Departamento de Ciencias de la Salud, División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Ciudad de México, Mexico

[2]Departamento de Ciencias de la Salud, División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Ciudad de México, Mexico

[3]Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México, Ciudad de México, Mexico

ABSTRACT

Background: Severe malnutrition is the main cause of immunodeficiency in children under 5 years in areas of economic depression in developing countries, and it is associated with generalized atrophy of lymphoid tissues. *Urtica dioica* L. is a medicinal herb with multiple pharmacological functions; it acts as modulator of the immune response, stimulating the proliferation of T lymphocytes *in vitro*.

Objective: To determine the ability of *U. dioica* L. to modulate the immune response of malnourished rats in the first 21 days of life.

Methods: Third-degree malnutrition during lactation was induced by food competition, and treatment with *U. dioica* L. was tested. Day-old Wistar rats were randomly assigned to three groups: (a) the well-nourished group, (b) the untreated malnourished group, and (c) the malnourished group treated with 0.2 g/ml *U. dioica* L. on days 14, 16, 18, and 20. On day 21, animals were euthanized, and the thymus, spleen, bone marrow, and blood were obtained. Lymphocyte subpopulations were determined in the thymus and leukocyte subpopulations in blood. The total number of nucleated bone marrow cells was determined, and the proliferative capability of spleen lymphocytes from malnourished rats treated with *U. dioica* L. was compared to that of other groups.

Results: *Urtica dioica* L. decreased $CD4^-$ $CD8^-$ lymphocytes and increased $CD4^+$ $CD8^+$ lymphocytes in the thymus, while in the blood, it increased $CD4^+$ cells, monocytes, and total T lymphocytes. The proliferation of spleen cells was stimulated in the malnourished rats, and a decrease in total nucleated bone marrow cells was observed. An increase in the red blood cell count, hemoglobin, and white blood cell count was observed in malnourished rats treated with *Urtica dioica* L. ($p \leq 0.05$).

Conclusion: *Urtica dioica* L. had immunomodulatory effects since it promoted the differentiation of $CD4^-$ $CD8^-$ T lymphocytes toward $CD4^+$ $CD8^+$ T lymphocytes and counteracted anemia and leucopenia associated with severe malnutrition.

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Introduction

Malnutrition is a public health problem that mainly affects developing countries as a consequence of poor economic and social conditions. Malnutrition results from an imbalance between food intake and nutritional requirements [1–3]. Severe malnutrition or protein energy malnutrition (PEM) is an extreme condition and is the main cause of immunodeficiency in children under 5 years [4]. In neonates and preschool children, PEM has been shown to impair the immune response and is associated with generalized atrophy of lymphoid tissues [5,6], causing a chain of immunological alterations characterized by leucopenia, a decrease in the ratio of T $CD4^+/CD8^+$ lymphocytes and an increase in T

Contact Sara Beatriz Herrera-Solís ✉ sarahs@xanum.uam.mx Laboratorio de Hematología Experimental, Departamento de Ciencias de la Salud, División de Ciencias Biológicas y de la Salud-Iztapalapa, Universidad Autónoma Metropolitana, Ciudad de México, Mexico.

$CD4^-/CD8^-$ lymphocytes in circulating blood [7], anemia, and thrombocytopenia.

Different substances, such as flavonoids, polysaccharides, lactones, and glycosides, are present in several plants and can affect humoral or cellular factors of the immune response [8,9]. Plants such as *Panax ginseng*, *Tinospora cordifolia*, and *Asparagus racemosus* have been shown to stimulate the immune system [10] and, therefore, have opened doors for the development of new plant-based immunostimulatory drugs. Stinging nettle, *Urtica dioica* L., has been proposed to have such immunostimulatory effects.

Urtica dioica L. (Urticaceae) is an herbaceous plant of economic importance due to its medicinal, food, nutritional complement, and textile properties. This plant is widely distributed in America, Europe, and Asia [11,12]. *Urtica* grows in central and Southwestern Mexico, where it is known by many common names, including "Ortiga," "Chichicastle," and "Mala mujer" [13,14]. It is an annual or perennial plant that reaches up to 1 m in height and presents stinging trichomes on the stem and leaves.

Urtica dioica L. has been recommended as an adjuvant treatment for rheumatic conditions, a nutritional tonic, and anti-anemic treatment, and has been suggested for the treatment of headache, eczema, skin care, edema, lower urinary tract infections, and allergies [15–17]. *Urtica dioica* L. has multiple pharmacological functions, including anti-inflammatory, analgesic, antioxidant, antiplatelet, antihyperglycemic, antihyperlipidemic, antiviral, anticancer, and antiandrogenic activities, among others [18].

In vitro studies have shown that the plant possesses β-sitosterol and scopoletin, molecules that could be of clinical use in the management of benign prostatic hyperplasia because both inhibit 5α-reductase, which is associated with this condition [19]. As mentioned previously, another important activity of *U. dioica* L. is its role as a modulator of the immune response because it has been reported that *U. dioica* L. increases chemotaxis and the metabolic integrity of neutrophils from healthy humans *in vitro* [20]. Quercetin and two glycosidic flavonoids from a methanolic extract of the aerial parts of this plant stimulated chemotaxis of neutrophils [21]. In addition, *U. dioica* L. has been shown to stimulate the proliferation of mouse spleen T lymphocytes *in vitro* and result in a moderate increase in $CD4^+$ T lymphocytes [22]. An aqueous decoction of the aerial structures of the plant stimulated the proliferation of mouse spleen lymphocytes *in vitro*, thereby confirming that the plant extract can stimulate proliferation of cells of the immune system [23].

Urtica dioica L. has a wide range of compounds, including glycopyranosides, glycoproteins, proteins, flavonol glycosides, carotenoids, and biologically active compounds, such as caffeic acid, quinic acid, essential oils, formic and acetic acid, histamine, tannins, mucilage, and vitamins (A, B1, B2, C, K1, folic acid, and pantothenic acid), which can contribute to improved health in childhood malnutrition [24,25].

Considering that severe malnutrition causes immunodeficiency in children under 5 years of age, mainly in developing countries such as Mexico, which is rich in plants for medicinal use, among them *U. dioica* L.; the present study aimed to determine the effects of *U. dioica* L. on the proportion of T and B lymphocytes and the proliferative capacity of thymus, spleen, bone marrow, and blood cells using an experimental model of rats with third-degree malnutrition.

Materials and Methods

Chemicals and reagent tests

The following chemical and reagent tests were obtained: xylazine [Procin, México; Chemical Abstracts Service (CAS) 23076-35-9]; sodium chloride (NaCl; Meyer, México; CAS 7647-14-5); phosphate-buffered saline (PBS-DULBECCO; Microlab, Mexico; Mecanismo de Desarrollo Limpio MFCD00131855); monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, and anti-CD45 (Biolegend, San Diego, CA); fluorochromes [fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC)] (BD Biosciences, San Jose, CA); Cell Counting Kit-8 (CCK-8, Dojindo Corp., Japan); trypan blue (CAS 72-57-1); Concanavalin A (ConA; CAS 11028-71-0); bovine serum albumin (Cohn Fraction V, pH 7.0; CAS 9048-46-8); and paraformaldehyde (CAS 30525-89-4, Sigma Chemicals Co., St. Louis, MO).

Stinging nettle (*U. dioica* L.) leaf extract (Enzymatic Therapy, LLC, part of Nature's Way Brands) was obtained. Stinging nettle (600 mg, serving size 2 capsules) was standardized to contain 1% silicic acid. This product contains natural ingredients. Entreprise de Taille Intermédiaire is a The Food and Drug Administration-registered drug establishment.

Determination of the total polyphenol content of the extract of U. dioica *L.*

The total concentration of phenols was determined by spectrophotometry using Folin–Ciocalteu reagent as the oxidant agent [26].

Calibration curve

A standard solution of gallic acid (0.1 mg/ml) was used, from which volumes were taken from 0 to 100 µl in 20 µl intervals, and the volume of each solution was diluted up to 500 µl with distilled water. Determination of phenols in the powder of a capsule of *U. dioica* L. was performed. First, 5 mg of extract was dissolved in 1 ml of distilled water and diluted 1:10 with distilled water, and 40 µl of this solution was taken and then diluted to 500 µl with distilled water. Finally, to each of the standards and previously prepared plant sample, 250 µl of Folin–Ciocalteu 1 N reagent was added and agitated in a vortex for 5 minutes. Subsequently, 1,250 µl of 20% Na_2CO_3 was added and allowed to stand for 2 hours. The absorbance was measured at 760 nm. The results are expressed as a percentage of gallic acid equivalent per milligram dry matter (% GAE/mg dry matter).

Experimental animals

Wistar rats were obtained from the animal facilities of the "División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana-Iztapalapa (UAM-I)" in Mexico City, Mexico. The animals were kept under controlled temperature (22°C–25°C) and 45% relative humidity with a 12–12 hour light–dark period (light 07:00–19:00 hour) with free access to food (Purina Mills International 5001, Richmond, VA) and water. Experiments were performed according to the guidelines of the UAM-I and the Official Mexican Guidelines (NOM-062-ZOO-1999). Nursing mother rats of approximately 350 g at 4 months of age were used.

Induction of malnutrition and treatment U. dioica *L.*

Malnutrition during lactation was induced by food competition [27]. Rats (1 day of birth) from different litters were randomly assigned to one of two groups: (1) the well-nourished group (WN), eight pups with a nursing mother (control group), and (2) the malnourished group (MN), 16 pups with a nursing mother.

The pups were weighed every 2 or 3 days to estimate the body weight of each litter from the first day until weaning (day 21) to determine the degree of malnutrition compared to age-matched controls (WN). On day 14, rats with a weight deficit greater than 40% (rats with third degree or severe malnutrition) were used. Then, the MN group was distributed into two subgroups: (a) MN rats that received saline physiological solution and (b) MN rats treated with 0.2 g/ml *U. dioica* L. (MNUd), which was intragastrically administered through a cannula on days 14, 16, 18, and 20. On day 21, rats were anesthetized with xylazine, and blood was obtained by cardiac puncture with a heparinized syringe. Thymus, spleen and bone marrow were obtained. The weight (in grams) of the thymus and spleen of each rat was recorded and compared with the WN group. For the comparison, one-way analysis of variance (one-way ANOVA) was employed with the Tukey test as necessary to establish the statistical significance, for which 95% was taken as an index of reliability with a $p < 0.05$.

Determination of lymphocyte subpopulations in thymus

The thymus was extracted, cells were obtained by homogenizing the organ and filtering through 85-mm mesh, and the disrupted cells were suspended in Ca^{++} and Mg^{++}-free phosphate saline (PBS, pH 7.0). Cell viability was determined with 0.2% trypan blue and adjusted to 1×10^7 cells/ml, which were placed in 5 ml tubes (Falcon®). Cells were stained with 2 µl of monoclonal antibodies, anti-CD4 and anti-CD8 conjugated to APC and PE fluorochromes, for 30 minutes. Then, 2 ml of 0.5% PBS-albumin was added and centrifuged for 5 minutes at 1,500 rpm. Subsequently, 500 µl of fixative solution (1% paraformaldehyde) was added. Ten thousand cells were analyzed on a flow cytometer using the CELLQuest® program. The results are expressed as the percentage of positive cells for the corresponding marker. In each sample, $CD4^+$ $CD8^+$ (double positive, for both fluorescence markers), $CD4^-$ $CD8^-$ (double negative, for both fluorescence markers), $CD4^+$ $CD8^-$ (single positive, only for CD4 fluorescence), and $CD8^+$ $CD4^-$ (single positive, only for CD8 fluorescence) cells were identified.

Determination of leukocyte subpopulations in blood

A total of 100 µl of blood from each rat was loaded into 5 ml tubes (Falcon®), and 2 µl of the following monoclonal antibodies conjugated to different fluorochromes were used: (1) $CD3^+$ FITC, $CD8^-$ PE, and $CD4^+$ APC for CD4 T lymphocytes, (2) $CD3^+$ FITC, $CD8^+$ PE, and $CD4^-$ APC for CD8 T lymphocytes, (3) $CD3^-$ FITC, $CD8^-$ PE, and $CD4^+$ APC for monocytes, (4) CD45-RA FITC and $CD45^+$ PerCP for B lymphocytes, and (5) $CD3^+$ FITC and $CD45^+$ PerCP for total T lymphocytes. After 30 minutes, 2 ml of lysis solution (FACS Lysing Solution, Becton Dickinson Immunocytometry Systems, San Jose,

CA) was added and incubated for 10 minutes at room temperature. Subsequently, each sample was centrifuged for 5 minutes at 500 g at room temperature, the supernatant was removed, and the cell button was washed with 0.5% PBS-albumin. Then, 500 µl of the paraformaldehyde fixative solution (1% in PBS) was added, and the cells were suspended prior to determination. At least 10,000 cells were analyzed by flow cytometry. The analysis was performed with CELLQuest®. The results were analyzed in dot graphs and are expressed as the percentage of positive cells for each marker. For each sample, four cell types were identified: $CD4^+$ $CD8^-$ cells (single positive, only for CD4 fluorescence), $CD8^+$ $CD4^-$ cells (single positive, only for CD8 fluorescence), monocytes, B lymphocytes and T lymphocytes. Granulocytes were not determined.

Flow cytometry

Determination of lymphocyte subpopulations in the thymus and determination of leukocyte subpopulations in blood was performed with a FACSCalibur flow cytometer (Becton Dickinson, Immunocytometry System (BDIS), CA) with an argon laser (488 nm).

Automated blood cell analysis

The total concentration of white blood cells (WBCs), red blood cells (RBCs), erythroid parameters, platelet count, and the lymphocyte/neutrophil relationship were determined using a KX-21N automatic analyzer (Sysmex, Japan).

Total count of nucleated bone marrow cells

The right femur of each rat was dissected, their muscles were removed and 1 ml of physiological saline was injected through the femoral canal. The cells were collected in a plastic tube and homogenized by pipetting. The cell suspension was diluted 1:20 with Turk's solution, and total nucleated cells were counted in a Neubauer chamber under a light microscope.

Determination of proliferation of spleen lymphocytes from malnourished rats treated with U. dioica *L.*

Under sterile conditions, the spleen of rats was isolated, dispersed mechanically on a metal 30 µm mesh, and washed with saline solutions. The cell suspension with 95% viability was added (trypan blue 0.2%) and adjusted to 2×10^6/ml in 96-well plates containing 100 µl aliquots in the presence of 10 mg/ml ConA and incubated at 37°C in an atmosphere of 5% CO_2 and humidity of 90% for 68 hours. As a control group, cells were cultured with RPMI-1640 medium without ConA. Each culture was performed in triplicate five times. Subsequently, 10 µl of CCK-8 (CCK-8, Dojindo Corp., Japan) was added to each well, and the plate was incubated for 4 hours at 37°C in a 5% CO_2 and 90% humidity atmosphere. The absorbance at 450 nm was read with an enzyme-linked immuno sorbent assay plate reader (BioTek, USA).

Statistical analysis

The statistical program NCSS 2010 (Version: 07.1.20) was used. Values are expressed as the mean ± standard error (M ± SE). For the comparison between the means of the treated groups *versus* the control groups, we employed one-way ANOVA with Tukey's test and the Duncan test as necessary to establish statistical significance, using 95% as an index of reliability with a $p < 0.05$.

Results

Preliminary determination of total polyphenols in the extract of U. dioica *L.*

The concentration was calculated from a standard curve ($y = 0.0674X + 0.0082$; $R^2 = 0.9975$) made with gallic acid. The content of total polyphenols in *U. dioica* L. capsules was 2.43% GAE/mg dry matter.

Body weight of well-nourished and experimentally malnourished rats left untreated and treated with U. dioica *L. during the lactation period*

Through the model of malnutrition, only 30%–40% of rats per litter reached the third degree of malnutrition; the cumulative data are shown in Table 1.

The average body weight of newborn rats (before the treatment) was 7.6 ± 0.1 g. At day 20, the average body weight of the WN group was 43.4 ± 0.6 g *versus* 26.1 ± 0.3 g in the MN group and 26.0 ± 0.7 g in the MNUd group. MN rats had a weight ten times lower than the WN rats, indicating that the MN and MNUd groups were still malnourished. In addition, no statistically significant difference was observed between the weights of the MNUd rats and the untreated MN rats (Table 1).

Weights of the thymus and spleen

The average weight of the thymus in the WN rats was 0.145 ± 0.017 g, whereas the average weight

Table 1. Body weight observed during lactation in well-nourished rats and malnourished rats.

Group	Day								
	1	4	7	9	12	14	16	18	20
WN	7.6 ± 0.1	11.0 ± 0.4	14.8 ± 0.5	20.1 ± 0.5	24.8 ± 0.5	29.6 ± 0.5	33.8 ± 0.5	38.1 ± 0.6	43.4 ± 0.6
MN	7.6 ± 0.1	9.2 ± 0.1	11.5 ± 0.1	14.8 ± 0.1	16.9 ± 0.2*	20.3 ± 0.2*	22.1 ± 0.2*	24.2 ± 0.3*	26.1 ± 0.3*
MNUd	7.6 ± 0.1	9.2 ± 0.1	11.5 ± 0.1	14.8 ± 0.1	16.9 ± 0.2*	19.6 ± 0.4*	21.8 ± 0.4*	23.9 ± 0.6*	26.0 ± 0.7*

WN = well-nourished rats (n = 68), MN = malnourished rats (n = 161), and MNUd (n = 37) = Malnourished rats treated with *Urtica dioica* L. Data are expressed as the mean ± SE. *p < 0.05, significant differences with respect to the WN group.

in the MN rats was 0.050 ± 0.007 g, and the average weight in the MNUd rats was 0.069 ± 0.008 g, indicating that no significant difference was observed among the malnourished groups. However, the WN group had a significantly higher value than the malnourished groups. In addition, no significant difference was observed in the total body weights of both malnourished groups.

The mean spleen weights for the WN, MN, and MNUd groups were 0.227 ± 0.017 g, 0.067 ± 0.007 g, and 0.090 ± 0.010 g, respectively. The spleen weight of the MN group was 70% less than the spleen weight of the WN rats, and the mean spleen weight of the MNUd rats was 60%, indicating that the spleen weight of this group gained 10% (Fig. 1).

Thymocyte subpopulations

Rats from the MN group had a higher average percentage of $CD4^-$ $CD8^-$ thymocytes than rats from the other groups (3.04 and 1.8 times higher than the WN and MNUd group values, respectively), and the differences were statistically significant at $p < 0.05$. In contrast, the MN group presented a lower average percentage of $CD4^+$ $CD8^+$ than the other groups. No significant differences were observed between the WN and MNUd groups (Fig. 2).

Distribution of circulating white blood cells

The mean percentages of $CD4^+$ and $CD8^+$ T lymphocytes and monocytes were lower in the MN group than in the WN group, except for B lymphocytes. The proportion of leukocytes in MNUd rats was similar to that in WN rats, except for T cells, which were decreased. There was a significant difference between the WN and MN groups; thus, malnutrition decreases the proportion of leukocytes. Although the MNUd group showed a higher proportion of $CD4^+$ cells, $CD8^+$ T lymphocytes, monocytes, and B cells than the MN group, only the $CD4^+$ and monocyte counts showed statistically significant differences between the groups (Fig. 3).

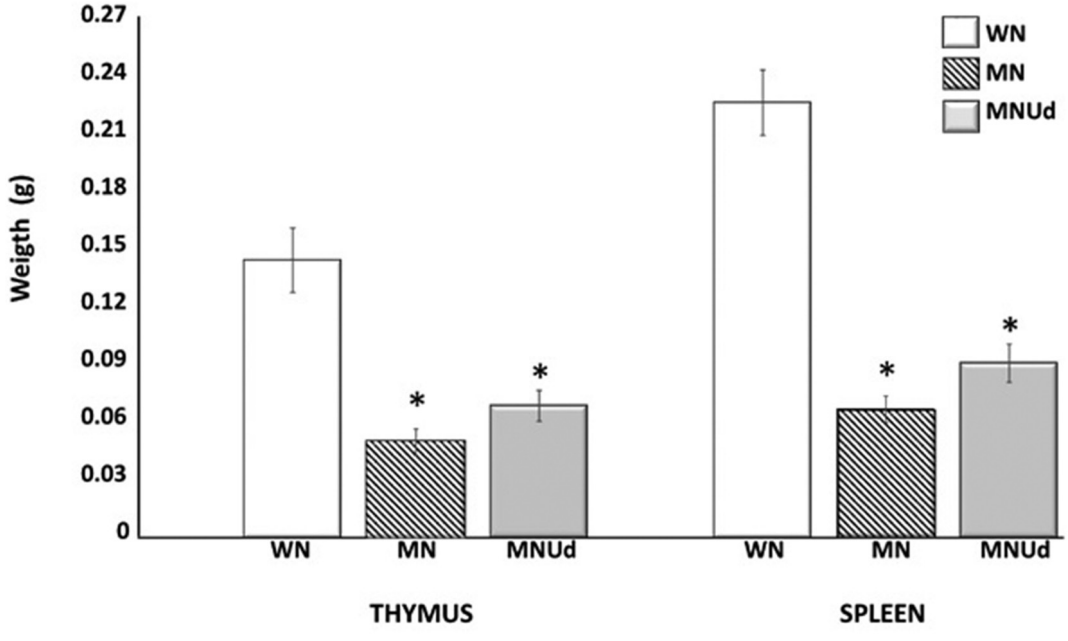

Figure 1. Weight of the thymus and spleen of malnourished rats. WN—Well-nourished rats (n = 10); MN—Malnourished rats (n = 11); and MNUd—Malnourished rats treated with *Urtica dioica* L. (n = 15). Data are expressed as the mean ± SE. *p < 0.05, significant differences with respect to the WN group.

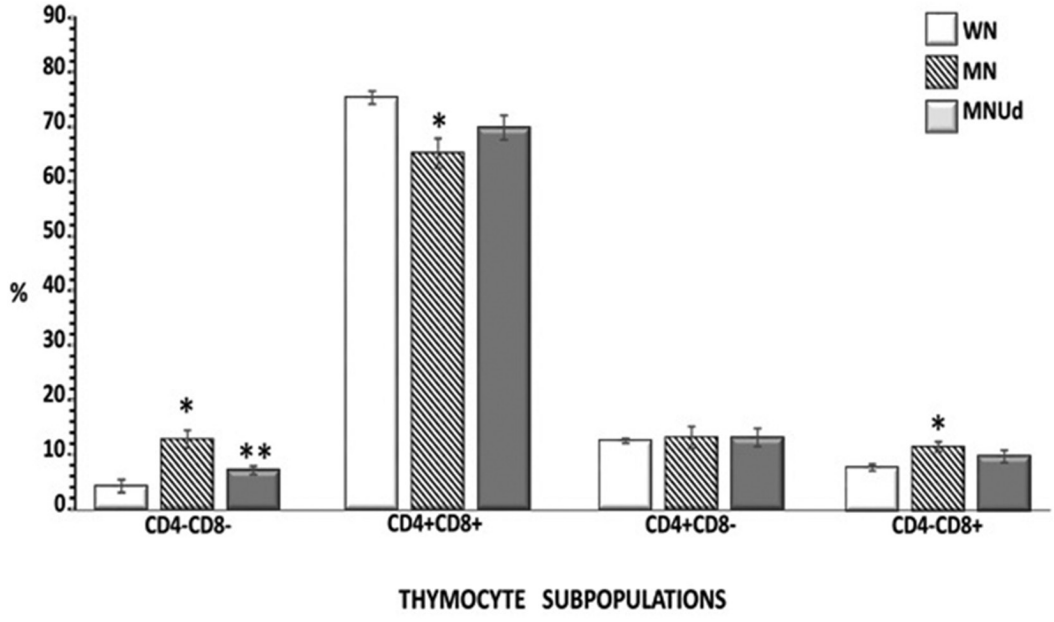

Figure 2. Average percentage of thymocyte subpopulations of malnourished rats treated with *Urtica dioica* L. WN—Well-nourished rats (n = 5); MN—Malnourished rats (n = 5); and MNUd—Malnourished rats treated with *Urtica dioica* L. (n = 5). Data are expressed as the mean ± SE. *$p < 0.05$, significant differences with respect to the WN group. **$p < 0.05$, significant differences with respect to the MN group.

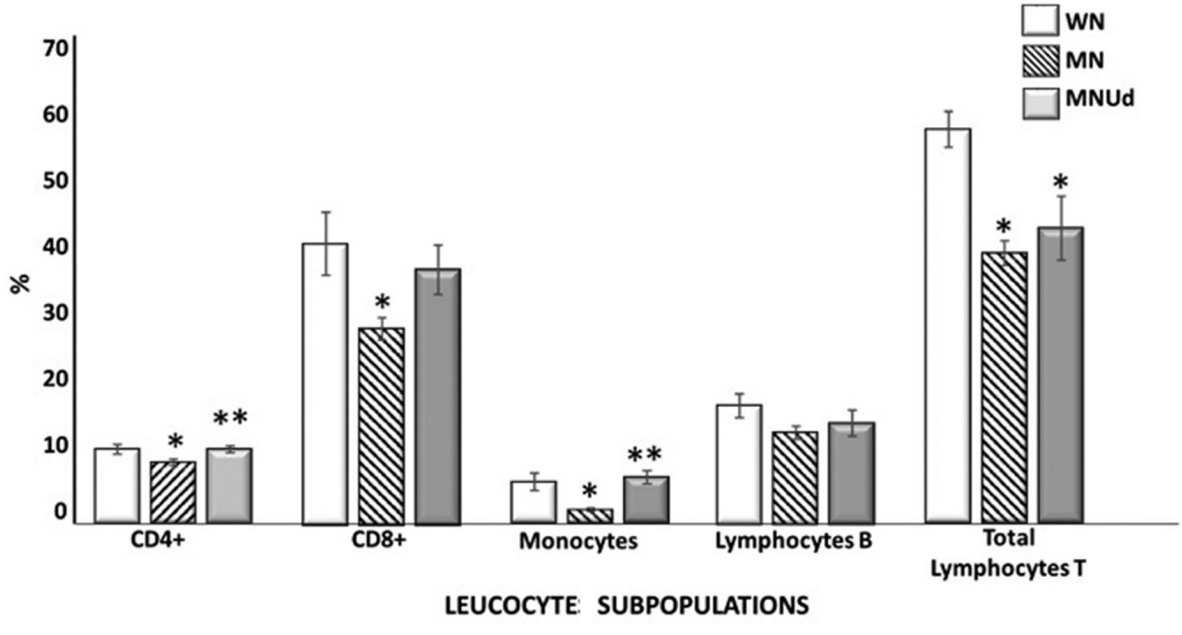

Figure 3. Average percentage of circulating WBCs in rats treated with *Urtica dioica* L. WN—Well-nourished rats (n = 5); MN—Malnourished rats (n = 10); and MNUd—Malnourished rats treated with *Urtica dioica* L. (n = 10). Data are expressed as the mean ± SE. *$p < 0.05$, significant differences with respect to the WN group. **$p < 0.05$, significant differences with respect to the MN group.

Hemocytometry

The concentration of WBCs in the MN group decreased by 29% and 25% compared to that of the WN and MNUd groups, respectively. RBC concentrations and erythroid parameters, including hemoglobin, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), of the MNUd group tended to be higher than those of the MN group. However, no

Table 2. Hemocytometry of malnourished rats treated with *Urtica dioica* L.

Group (*n*)	WBC × 10^3/µl	RBC × 10^6/µl	Hb (g/dl)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT × 10^3/µL
WN (10)	4.2 ± 0.6	5.3 ± 0.2	9.3 ± 0.4	31.3 ± 1.6	57.5 ± 1.6	17.0 ± 0.4	29.7 ± 0.7	408.8 ± 72.3
MN (9)	3.0 ± 0.2*	4.1 ± 0.4*	8.4 ± 0.6	27.0 ± 2.0	58.7 ± 2.0	19.0 ± 0.7	31.3 ± 0.4	341.0 ± 46.9
MNUd (11)	4.0 ± 0.4	4.7 ± 0.3	9.3 ± 0.6	29.3 ± 1.7	61.9 ± 0.9	20.2 ± 0.6*	32.6 ± 0.7*	290.4 ± 55.1

Hb = hemoglobin; PLT = platelet.
WN = well-nourished rats (*n* = 10), MN = malnourished rats (*n* = 9), and MNUd = malnourished rats treated with *Urtica dioica* L. (*n* = 11). Data are expressed as the mean ± SE.
*$p < 0.05$, significant differences with respect to the WN group.

statistically significant differences between the WN and MNUd groups were observed, except for MCH and MCHC, which were higher in the MNUd group ($p \leq 0.5$) (Table 2).

The ratio of lymphocytes/neutrophils in the MN and MNUd groups showed a reduction of lymphocytes with respect to that of the WN group. However, this reduction was not statistically significant. Neutrophils increased in the MN and MNUd groups compared with the WN group (Fig. 4).

Nucleated bone marrow cells

No statistically significant differences were observed among the WN, MN, and MNUd groups (Fig. 5).

Spleen cell cultures

In the WN group exposed to ConA, there was a 19.1% increase in the absorbance, indicating increased cell proliferation with respect to the same culture without ConA. For the MN group, in the presence of ConA, the increase in absorbance was 9.2%. The MNUd group plus ConA showed an increase of 41.1% compared with the same group without ConA (Fig. 6).

Discussion

In previous studies, researchers looking for *U. dioica* L. in the field collected plants with similar morphology that turned out to be *U. chamaedryoides* and *U. dioica* var. Angustifolia, which showed an ability to restore hematopoiesis in pregnant mice, preventing the development of malformations in fetuses [28,29]. In later collections, the specimens were identified as *U. urens*. In addition, aqueous extract of the leaf of *U. dioica* L. (capsules) commercialized as a nutritional supplement was purchased, and a comparative study was conducted between an aqueous extract of *U. urens* and the capsules of *U. dioica* L. to assess the ability of both to stimulate the

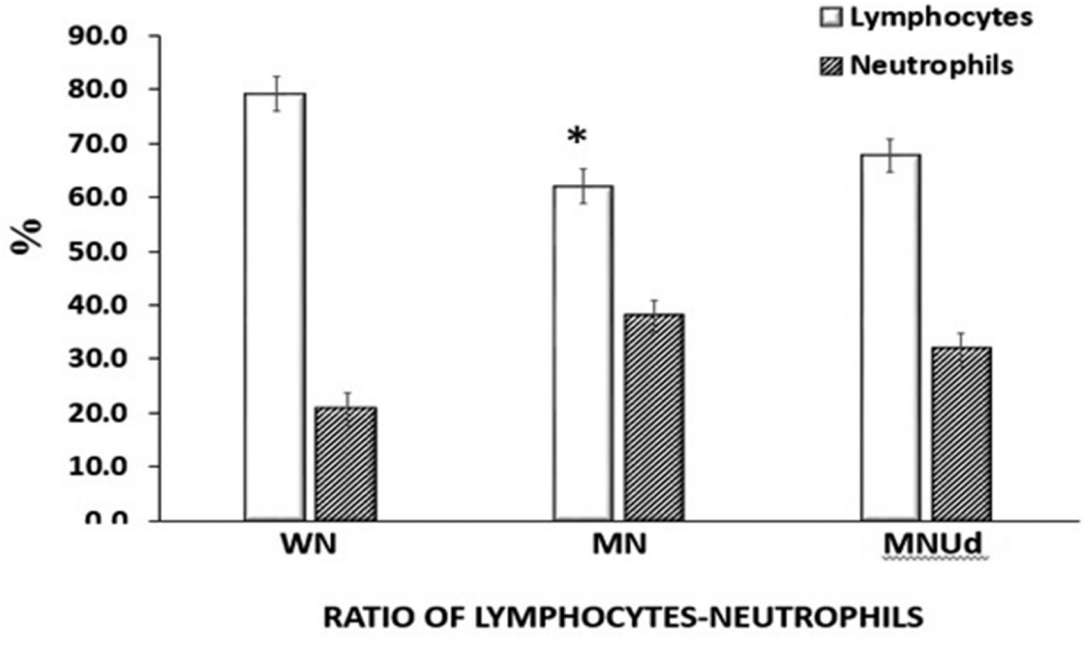

Figure 4. Lymphocyte-neutrophil count ratio of malnourished rats. WN—Well-nourished rats (*n* = 10); MN—Malnourished rats (*n* = 9); and MNUd—Malnourished rats treated with *Urtica dioica* L. (*n* = 11). Data are expressed as the mean ± SE. *$p < 0.5$, significant differences with respect to the WN group.

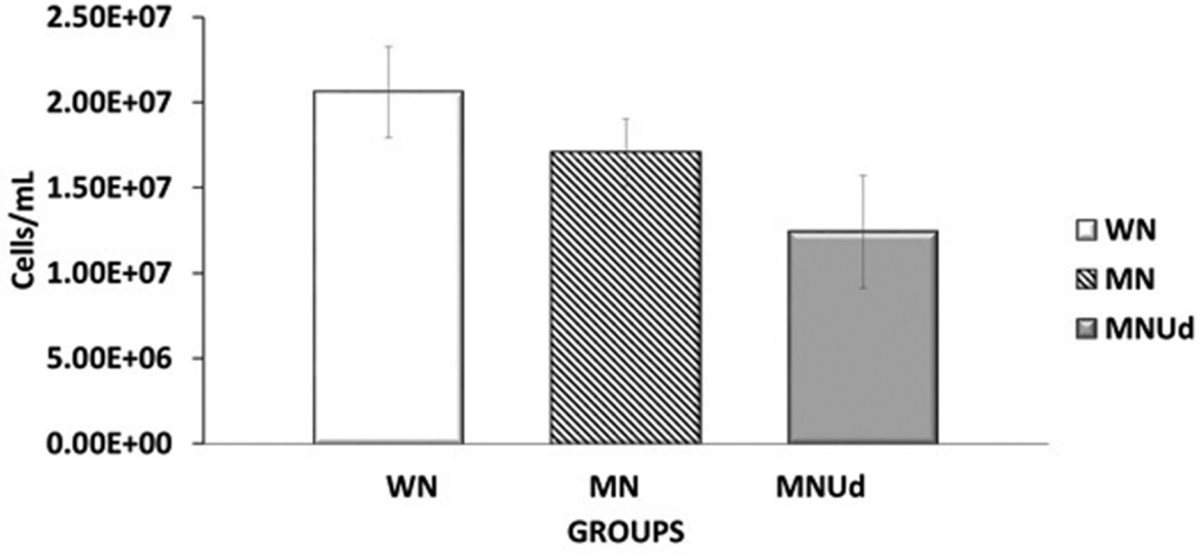

Figure 5. Total nucleated cells of bone marrow from malnourished rats. WN—Well-nourished rats (n = 10); MN—Malnourished rats (n = 10); and MNUd—Malnourished rats treated with *Urtica dioica* L. (n = 10). Data are expressed as the mean ± SE. *p < 0.5, significant differences with respect to the WN group.

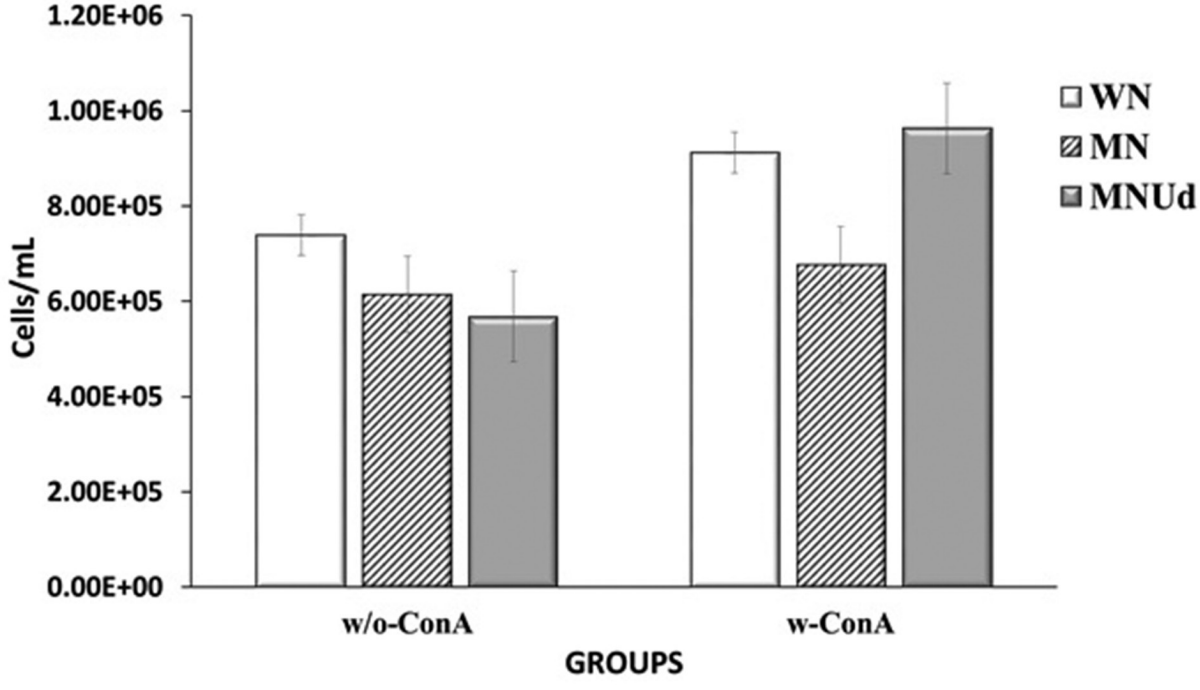

Figure 6. Proliferation of spleen cells from malnourished rats. The cultures were made in the absence (w/o-ConA) and presence of Concanavalin A (w-ConA). WN—Well-nourished rats (n = 15); MN—Malnourished rats (n = 8); and MNUd—Malnourished rats treated with *Urtica dioica* L. (n = 10). Data are expressed as the mean ± SE. *p < 0.05, significant differences with respect to the WN group.

proliferation of rat spleen lymphocytes *in vitro*. The highest and constant activity was obtained with the *U. dioica* L. capsules, and for this reason, after establishing a dose response curve (results not included), the present work was performed with *U. dioica* L. capsules.

The concentration of phenolic compounds in capsules of stinging nettle (*U. dioica* L.) leaf extract was 2.43% of dry matter by weight; this is an important finding with respect to the different biological properties of these metabolites and their possible application in various areas, such as food/feed,

cosmetics, phytomedicine, and textiles. However, it is important to note that phenolic compounds were determined only as reference due to their normal presence in the plant.

Our results showed that severe malnutrition was induced in Wistar rats by food competition, and the MN and MNUd groups presented a 40% body weight deficit, in addition to other signs, such as poor hair, brittle bones, and poor motor activity similar to those observed in malnourished children [30]. At the end of the experiment, the MN and MNUd groups presented reduced thymus and spleen weights and severe atrophy of the thymus [31], indicating that the *U. dioica* L. promoted cell proliferation, although it was not sufficient to increase the levels to the WN group levels.

Severe protein malnutrition causes leucopenia, a decrease in the proportion of $CD4^+$ and $CD8^+$ T lymphocytes, and an increase in the number of $CD4^-$ $CD8^-$ cells, plus immature T cells in the circulation [32,33], as we observed in the MN group. In the MNUd group, there was a restoration of hematopoietic cell levels, indicating that our extract may promote cell differentiation and maturation to almost normal levels. The data showed that treatment with *U. dioica* L. significantly increased the percentage of $CD4^+$ cells, monocytes, and T lymphocytes, resulting in cell counts in the MNUd group that did not significantly differ from those of the WN group. These results are in agreement with those reported by other authors regarding the capacity of *U. dioica* agglutinin (UDA) to stimulate the proliferation of T lymphocytes, although it does so in a delayed manner with respect to ConA [34]. The UDA behaves like a superantigen that activates the Vβ segments of the T lymphocyte receptor (TCR) by binding to molecules of the major histocompatibility complex type I and II [35]. Notably, the aforementioned agglutinin has been isolated from the rhizome of the plant.

Our study was carried out with a crude extract of the leaf that does not allow us to assume that this is the same mechanism of stimulation as that for the proliferation of T cells [36].

In blood cytometry analyses, the concentration of WBCs in the MN group was lower by 29% and 25% compared with that of the WN and MNUd groups, respectively. However, there were no significant differences between the WN and MNUd groups. Differences in circulating blood parameters between the MN and MNUd groups can be attributed to the plant extract because there were 7 days from the first administration of *U. dioica* L. to the sacrifice of the animals, a period that matches the term of 5–7 days average for the production of blood cells. That is, treatment with the plant extract in the MNUd group promoted the recovery of hematopoiesis, while in the untreated MN group, this recovery was not observed even with adequate time [37]. The total nucleated bone marrow cells were lower in the MNUd group than in the MN group, probably because the plant promotes the rapid release of cells from the bone marrow to the blood.

In Mexican traditional medicine, *U. dioica* L. is used to treat anemia due to its high iron content, which may contribute to the increase in erythrocytes and specifically to the mean corpuscular hemoglobin concentration. A comparable finding was observed when a decoction of *U. dioica* L. var. angustifolia prevented malformations in the fetuses of anemic mice, although the mother remained anemic [29]. Nettle plants are known to be very good sources of energy, proteins, fiber, and a range of bioactive compounds that have health benefits [38]. In Nepal, *U. dioica* L. is both a staple food and supplementary food, which is often the top cash crop of local communities, contributing to food security in the Nepal region. In the case of Mexico, although it is a developing country rich in flora and fauna, severe PEM has a high incidence in suburban and rural areas [39]. The effects of *U. dioica* L. on rats with immunodeficiency due to severe malnutrition observed in this work can be attributed to the plant acting as a phytomedicine reinforced by its role as a nutraceutical.

The immunostimulatory role of *U. dioica* L. has been previously reported and identified as a property of the root. In our work, the activity was detected in the leaf. Notably, although it is already known that *U. dioica* L. stimulates the proliferation of T lymphocytes in healthy individuals, our results reinforce this knowledge but in a pathological condition, malnutrition, which is frequent in developing countries, where malnutrition is observed in a high percentage of the child population.

Conclusions

The aqueous extract of the leaves of *U. dioica* L. stimulates the immune response because it promotes the differentiation of T lymphocytes toward $CD4^+$ and $CD8^+$ cells, in addition to countering anemia and leucopenia associated with severe malnutrition. The effect of the nettle plant extract on lymphocyte activation and its production of cytokines is still being investigated.

Considering the pharmacological functions of *Urtica dioica* L. and its role as an anti-inflammatory, analgesic, antiandrogenic, antihyperglycemic, antihyperlipidemic, antiviral, anticancer, and antianemic plant and that the treatment of diseases with patented medicines is becoming increasingly expensive, we note that it is important to highlight the action of *U. dioica* L. to counteract severe malnutrition, especially when a high percentage of the world's population lives in poverty and extreme poverty.

This plant could be used as a nutritional supplement or a nutraceutical even in those populations without sufficient resources, as their diet lacks the nutrients provided by *U. dioica* L.

Conflict of interest

The authors declare that there are no conflicts of interest.

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