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In experimental animals, *Medicago sativa* ethanol leaf extract reduces pains and inflammation

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ABSTRACT

The well-known medical herb *Medicago sativa* has long been recommended as a pain reliever. In this work, the analgesic and anti-inflammatory properties of the leaf were examined in rodents. The analgesic effect of the leaf extract was screened in mice and rats using acetic acid-induced writhing, and formalin paw edema models; the anti-inflammatory properties of the methanol extract were investigated in mice and rats using xylene and carrageenan models at 200 mg/kg, 400 mg/kg, and 600 mg/kg. Acetylsalicylic acid (ASA), 150 mg/kg, and distilled water (20 mL/kg). In addition, phytochemical screening and oral acute toxicity testing were carried out on the leaf extract. At the studied dosage, the *M. sativa* leaf ethanol extract showed a dose dependent analgesic effect. At 150 mg/kg, acetylsalicylic acid (ASA) also showed a notable reduction in discomfort. The considerable reduction in the tested indices of pains indicates the analgesic and anti-inflammatory properties of *M. sativa* leaf extract. The results suggest a possible direction for more research into *Medicago sativa* as a natural anti-inflammatory and analgesic.

Keywords: *Medicago sativa*; Analgesic; Inflammation; Ethanol leaf extract; Rodent models

1. INTRODUCTION

According to inflammation is a complex biological reaction to damaging stimuli, such as infections, injured cells, or irritants, which may result in eliminating the source of cell injury and starting tissue healing. However, a number of illnesses, including cancer, cardiovascular disease, and autoimmune disorders, can be brought on by persistent inflammation. The anti-inflammatory qualities of medicinal plants have long been reported, making them attractive candidates for the creation of therapeutic drugs. Majority of local population uses herbs to cure a variety of illnesses. Herbal traditions served as a source of modern medicine (Ezeonwumelu et al., 2012). Pain can range from mild to severe, intense, scorching, or dull, and it can be temporary, sporadic, or continuous. It can also take several forms. According to Hassan et al., (2015), pain can also be localized or distributed, superficially or deep.

Many psychological processes, including those related to attention, memory or learning, thought, beliefs, emotions, behavioral reactions, and coping capacity, might impact how someone perceives pain (Macintyre and Walker, 2010). A crucial component of healthcare is pain management, and scientists are always looking for new drugs and treatment approaches to alleviate pain. Examining natural compounds that may have analgesic and anti-inflammatory effects is one such line of inquiry. Alfalfa, sometimes known as *Medicago sativa*, is a plant in the Fabaceae family. *M. sativa* is a functional food and a medicinal plant with variety of pharmacological properties. This plant is known as a nutritional supplement and herbal medicine because of its therapeutic qualities and nutrients (Mikaili and Shayegh, 2011). Research on laboratory animals has shown that *M. sativa* accelerates the regeneration of joint cartilage with a positive impact on tissue repair (Mikaili and Shayegh, 2011). This study evaluated the analgesic and anti-inflammatory properties of leaf extract from *Medicago sativa*in experimental animals.

2. MATERIALS AND METHODS

Gathering and Classifying Plants

In the Owerri West Local Government Area of Imo State, Nigeria, fields in Obinze and Eziobodo provided fresh leaves of *Medicago sativa*, also known as alfalfa. A sample specimen, MOUAU/ZEB/21/009 was placed in the university herbarium for reference after the plant was recognized and verified by Mr. Ibe Ndukwe, a taxonomist at the Department of Forestry, College of Environmental Sciences, Michael Okpara University of Agriculture, Umudike, Abia State.

Plant material extraction

After cutting the leaves into bits, they were allowed to air dry at room temperature before being ground into a powder using a Warring commercial blender. The coarse powder of *M. sativa* leaves, weighing eight hundred grams (800 g, was measured using a sensitive digital weighing scale). After the powder was soaked for 48 hours at room temperature in a flask filled with 80% ethanol (2.5 L w/v), and was agitated for two minutes. The resultant mixture was separated using Whatman (No.1) filter paper, concentrated using a rotary evaporator, and dried over a water bath to produce 30.45 g (4 % w/w) of greenish-colored dry extract. It was kept for additional analysis at four °C in a refrigerator. Afterward, the extract was reconstituted in distilled water to provide the required dosages of 400, 600, and 200 mg/kg of body weight. The following formula, derived from Ezirim et al., (2022), was used to compute the % yield:

Yield percentage is equal to (Weight (g) of residue (dry extract))/ (Weight (g)of ground-up powdered material) ×100/1

Experimental procedures

In this study, sixty Wistar rats of both male and female weighing 200 - 250 g, and Swiss mice also male and female (20–25 g) were used to carry out the work. The University of Nigeria, Nsukka's Department of Zoology, Faculty of Biological Sciences, provided the animals. The Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Abia State University, Uturu, Abia State, housed them at its animal house. Before the trial, the animals underwent a 14 days period of acclimation. The animals had an ordinary diet (Ladokun feeds, Ibadan) and unlimited access to water. Standard humidity, temperature, and a 12-hour light/dark cycle were applied to their upkeep. The National Institute of Health (1996) Guide for the Care and Use of Laboratory Animals was utilized.

Screening with phytochemicals

The approach outlined by Airaodion et al., (2019) qualitatively evaluated the phytoconstituents of the leaf extract.

Acute toxicity examination of the extract

The safety of the *M. sativa* leaf extract was assessed by measuring its LD5O, following the protocol described by (Lorke, 1983). Two stages of the investigation were examined on mice. In the first phase, nine mice divided into three groups with three each, and they were all randomly assigned. The mice were given the leaf extract orally at 10 mg/kg, 100 mg/kg, and 1000 mg/kg, respectively. The animals were observed for first four hours and the following twenty-four hours, for any signs of toxicity or death. The next four groups of one rat per cage were given the extract at 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg along with 20 mL/kg of distilled water in the second phase. We watched for evidence of death and toxicity in 72 hours and 24 hours, respectively.

Mice writhing in acetic acid

The animals were pre-treated under Okorie et al., (2024) description and split into five groups of six each. Group 1, acting as the control, was given 20 mL/kg of regular saline. *M. sativa* leaf extract was administered to group 2, 3, and 4 at 200 mg/kg, 400 mg/kg and 600 mg/kg body weight, respectively; group 5 received 150 mg/kg body weight of acetylsalicylic acid (ASA). Each treatment was facilitated by using orogastric cannula. After thirty (30) minutes of pre-treatment, each mouse received 0.7% of an acetic acid aqueous solution (20 ml/kg). Thirty minutes after treatment, the mice were taken into separate transparent Perspex viewing boxes, and the number of abdominal constrictions was counted. For both the extract and ASA groups, the % inhibitions of constrictions recorded as follows:

Test mean − control mean / control mean x 100 equals % inhibition.

Nociceptive reactions in rats given formalin

Using the procedure outlined by Akuodor et al., (2016), five groups of six rats each were carefully selected. They were allowed unlimited access to water during their 24-hour fast. Here is how they were pre-treated: Group 1 was given 2 milliliters of distilled water as a control. Group 5 received 150 mg/kg of acetylsalicylic acid (ASA), whereas group 2, 3, and 4 respectively received 200 mg/kg, 400 mg/kg, and 600 mg/kg of the extract intraperitonealy. All the groups received 50 μL of a 2.5% formalin solution subcutaneously (S.C.) under the left hind paw's subplanter surface thirty (30) minutes following the start of the treatment. After that, they were monitored under surveillance for 60 minutes in a chamber. The following scale was used to record the degree of nociceptive responses:

Rats can move freely and support their weight on an injected paw (0 =).

1 = The injected paw gently resting on the ground

Two = Partial paw elevation upon injection

Three = Complete paw elevation following injection.

Four = Biting or licking the injected paw.

Ear edema in mice induced by xylene

In each cage, the thirty Swiss mice were shared in five groups of six rats. Before the trial, the animals were given a 24-hour fast before being given unlimited access to water. Group 1 received 20 milliliters per kilogram of distilled water as a control. Group 2, 3, and 4 received 200 mg/kg, 400 mg/kg, and 600 mg/kg of *M. sativa* leaf extract, p.o., whereas group 5 received the conventional medication, dexamethasone (4 mg/kg). Each mouse had a drop of xylene applied to its inner right ear, which caused edema to develop one hour after administering the drug. After three hours, the animals were weight-matched and their ears chopped off to the same size while they were under halothane anesthesia for the sacrifice. Using the technique of inflammation was taken as the mean difference between the right and left ear for each group.

Wistar rats with paw oedema caused by carrageenan

With a slight modification, the methodology outlined by Okokon and Nwafor, (2010) was carried out to examine anti-inflammatory activity. The thirty Swiss rats were split into five groups of six and fasted for 48 hours, during which they were allowed unrestricted access to water. Group 2, 3, and 4 received the extract (200 mg/kg, 400 mg/kg, and 600 mg/kg body weight p.o., respectively), whereas Group 1 received a 0.5% carboxymethyl cellulose suspension (control). Group 5 received 150 mg/kg body weight of acetylsalicylic acid (ASA).

Following a one-hour pre-treatment with the extract, each animal received an injection of 0.05 ml of a 1% carrageenan suspension in normal saline into the sub-planter region of its left hind paw to cause edema. Initial measurements of paw edema were taken at 0, 1, 2, 3, 4, and 5 hours following carrageenan injection. The actual edema was calculated by comparing the initial and subsequent results with the control. The following method was used to compute the inhibition of inflammation: % inhibition = 100 (VC-VT/VC), where VC stands for the mean edema in the control group and VT for edema in the group receiving medication and extract treatment.

Analytical statistics

Using the statistical software for social sciences (SPSS version 20), the mean ± standard error of the mean (SEM) is shown together with the results of one-way analysis of variance (ANOVA) and Dunnett's post hoc test. A statistically significant difference in the mean was shown by $p < 0.05$.

3. RESULTS AND DISCUSSION

Phytochemical determination

Alkaloids, saponins, tannins, flavonoids, terpenoids, steroids, cardiac glycosides, and tannins were the phytochemicals found in the ethanol leaf extract of *M. sativa*; anthraquinones were not detected.

Acute toxicity study

There were no mortality or adverse responses seen at any of the levels tested. Every mouse was in good health and was kept busy both throughout and after the research time. As a result, the mice's oral acute toxicity result exceeded 5000 mg/kg.

Effect of ethanol leaf extract of *M. sativa* on acetic acid-induced writhing in mice

The ethanol extract's three dosages, which ranged from 48% to 91% each, demonstrated notable ($p<0.05$ and $p<0.01$) activity (Table 1). Oral administration of the conventional medication, aspirin, or acetylsalicylic acid, resulted in 97% pain inhibitions comparable to the extract and a substantial ($p<0.01$) decrease in writhing (Table 1).

Table 1 The ethanol leaf extract effect of *M. sativa* on acetic acid-induced writhing in mice.

Drug	Dose (mg/kg)	Writhes	% inhibition
Control	20 mL/kg	37.50±2.53	-
M. sativa	200	19.50±0.43	48a
M. sativa	400	8.83± 0.48	76b
M. sativa	600	3.50± 0.43	91b
Aspirin	150	1.00± 0.52	97 b

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

Effect of ethanol leaf extract of *M. sativa* on formalin-induced nociception in rats

Rats that were injected formalin to produce pain demonstrated dose-dependent analgesic effects. At the doses examined, as indicated in Table 2, there was a substantial ($p<0.05$) decrease in responses to noxious stimulus during the early, late, and ($p<0.05$ and $p<0.01$) phases of the ethanol leaf extract of *M. sativa*. Comparing aspirin to control, it also demonstrated a substantial ($p<0.01$) inhibitory response in both phases.

Table 2 Effect of ethanol leaf extract of *M. sativa* on formalin-induced pain in rats

Treatment groups	Early phase (0 to 10 min)		Late phase (15 to 60)	
	Score of pain	% Inhibition	Score of pain	% Inhibition
Control	2.9±0.08	–	2.83±0.08	–
200 mg/kg of *M. sativa*	1.44±0.12	49a	1.34±0.13	54a
400 mg/kg of *M. sativa*	1.23±0.11	57a	1.16±0.06	60a
600 mg/kg of *M. sativa*	1.04±0.51	64a	0.90±0.10	70b
150 mg/kg of Aspirin	0.90±0.09	70b	0.88±0.05	72b

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

Effect of M. sativa leaf extract on xylene-induced mouse ear edema

The xylene-induced mice showed topical edema in every ear. In all the dosage of the extract tested, there was an observed difference in the diameters of the edema between the untreated left ear and the treated right ear. The ethanol extract of *M. sativa* showed substantial ($p<0.05$ and $p<0.01$) inhibitory efficacy on the effect of the extract on acute edema (Table 3). Comparable to the standard medication dexamethasone, which was likewise significant at ($p<0.01$), is the anti-inflammatory activity.

Table 3 The effect of ethanol leaf extract of *M. sativa* on xylene-induced ear edema in mice

Treatment Groups	Weight of right ear (g)	Weight of left ear (g)	Increase in ear weight (g)	% Inhibition
Control	0.073±0.01	0.031±0.00	0.042±0.00	–
200 mg/kg of *M. sativa*	0.050±0.00	0.029±0.00	0.021±0.00	50a
400 mg/kg of *M. sativa*	0.038±0.00	0.025±0.00	0.013±0.00	70b
600 mg/kg of *M. sativa*	0.032±0.01	0.021±0.00	0.010±0.00	78b
4 mg/kg of Dexamethasone	0.029±0.00	0.020±0.00	0.009±0.00	80b

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

Effect of M. sativa leaf extract in ethanol on rats' carrageenan-induced paw edema

Table 4 displays the findings of the ethanol leaf extract of *M. sativa's* impact on rats' carrageenan-induced edema. It's worthy to note that the extract demonstrated considerable ($p<0.05$ and $p<0.01$) dose-dependent anti-inflammatory efficacy. Aspirin, the standard medication, likewise showed noteworthy ($p<0.01$) anti-inflammatory effect.

Table 4 Effect of M. sativa leaf extract against carrageenan-induced paw edema in rats

Time(hrs)								
Treatment	**Dose (mg/kg)**	**0**	**0.5**	**1**	**2**	**3**	**4**	**5**
Distilled water	20 mL/kg	1.28±0.03	1.64±0.03	1.82±0.03	1.71±0.03	1.88±0.03	1.96±0.02	2.40±0.03
M. sativa	200	1.22±0.03	1.64±0.02	1.55±0.02	1.42±0.02a	1.38±0.03	1.34±0.02a	1.26±0.02a
M. sativa	400	1.20±0.02	1.61±0.02	1.53±0.02	1.47±0.02a	1.41±0.03a	1.36±0.02a	1.22±0.02b
M. sativa	600	1.83±0.03	1.58±0.03	1.48±0.03	1.44±0.03a	1.32±0.04a	1.28±0.03a	1.19±0.03b
Aspirin	150	1.13±0.02	1.56±0.03	1.49±0.02	1.39±0.02a	1.30±0.02a	1.26±0.02a	1.10±0.02b

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

Since the experience of pain is regarded as one of the factors that lead individuals to seek medical attention, medications designed to alleviate pain have been widely approved (Akuodor et al., 2010; Mota et al., 2015). Due to side effects such as liver damage, the use of analgesics as pain relievers and killers, such as opiates, nonsteroidal anti-inflammatory medications (NSAIDs), and aspirin, has not always been effective (Zulfiker et al., 2010). Currently, the approach is to look for analgesics that are clinically novel, effective, and have minimal adverse effects. The analgesic effect of *M. sativa* was examined in rats by inducing writhing in response to acetic acid. In this experiment, pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) and prostaglandins, bradykinins, histamine, serotonin, and cyclooxygenase (COX) are stimulated in response to an intraperitoneal injection of acetic acid.

According to Ikeda et al., (2001) and Radu et al., (2013), these mediators penetrate the CNS's dorsal horn, trigger primary afferent nociceptors, and start the pain response. The current study showed that *M. sativa* ethanol leaf extract, at the levels used, had analgesic effects against acetic acid-induced pain that was chemically produced (writhing) (Sha'a et al., 2011). The extract with its peripheral analgesic qualities as seen by the results, shows that the effect may be due to local peritoneal nerve receptor opposition. The results confirmed the plant's value as an analgesic in Nigeria, regardless of whether the model assesses peripheral analgesic effect exclusively or non-specifically. According to Nkeh-Chungag et al., (2010), acetic acid injection produces prostaglandins and other cyclooxygenase mediators.

According to Nkeh-Chungag et al., (2010), this indicated that the ethanol extract of *M. sativa* worked by opposing the actions of cyclooxygenase, which is believed to promote prostaglandin synthesis through arachidonic acid. The experimental plant, *Medicago sativa* leaf, demonstrated a substantial analgesic effect; it prevented the animals in the experiment from writhing in response to acetic acid. The peritoneal fluid concentration of PG-E2 and PG-F2∧ was the cause of this pain mechanism, which is thought to engage local peritoneal receptors (Akuodor et al., 2010; Mota et al., 2015). (Tarkang et al., 2012). The animals responded by writhing, which is a distinctive stretching motion, and this approach is dependable and quick in assessing peripheral analgesic efficacy. Acetic acid-induced writhes are a sensitive method for determining analgesic impact of a drug (Akuodor et al., 2010).

It was obvious that *Medicago sativa* extract prevented the experimental animals' writhing reaction when exposed to acetic acid. The prostaglandin-induced sensitivity of pain receptors was associated to the aberrant constriction. It was plausible that the extract's analgesic effect resulted from its suppressing prostaglandin production. The formalin test consisted of two phases: The neurogenic phase, which occurred early due to direct stimulation of sensory nerve fibers, and the inflammatory phase, which occurred late from the release of chemical mediators like substance P, histamine, bradykinin, and serotonin (Patel and Dickenson, 2021). According to this investigation, the extract considerably lowered both phases compared to the control. Traditionally, it was reported that centrally acting medications markedly inhibited both early and late phases.

Consequently, inhibiting the test's early and late phases demonstrated that the *Medicago sativa* extract may have had both central and peripheral effects. The most accurate model for assessing clinical pain is the rat paw-licking tests generated by formalin, a chemical model of nociception that yielded a more focused response (Isiaka et al., 2019). Since the animals frequently utilized their forelegs during grooming, administering the irritating compound into the hind paw made the nociceptive reaction more specific (Choi et al., 2013). Two phases of nociceptive behavior were shown by the formalin-induced paw licking test, and these phases appeared to involve different mediators (Piccinelli et al., 2015). It was thought that direct chemical activation of nociceptors was the cause of the first nociceptive phase, which begins right after formalin injection and lasts for the following five minutes (Chakraborty et al., 2012).

Opioid agonists like morphine and fentanyl which antagonizes the bradykinin receptors, N-methyl-D-aspartic acid (NMDA) receptors, and vanilloid receptors, inhibited the first phase nociceptive. According to Avoseh et al., (2018), the second phase of this model occurred 15 to 30 minutes after the formalin injection and was associated with the release of many proinflammatory mediators. More so, both doses particularly the highest dose (600 mg/kg), significantly suppressed the formalin model's first and second phases. The finding of this study supports the anti-nociceptive and anti-inflammatory activities of *M. sativa* leaf extract Xylene is known to irritate mouse ears, resulting in fluid buildup and edema which is a hallmark of an acute inflammatory response (Akuodor et al., 2010). Suppression of this response may suggest an antiphlogistic effect. Xylene-induced ear edema was significantly and dose dependently inhibited by *Medicago sativa* leaf extract.

According to Caughey, (2016), this activity implies that the inhibition of phospholipase A2, which is involved in the pathophysiology of inflammation brought on by xylene. Flavonoids, saponins, tannins, tarpenoids, and alkaloids revealed in the extract may be responsible for its anti-inflammatory properties. The presence of one or more of the bioactive chemicals in the extract may have caused the dose-dependent inhibition to start. Flavonoids have shown to prevent the release of chemical mediators via histamine in rheumatoid arthritis, whereas serotonin alleviates symptoms. These are believed to be mediated by decreased fibroblast proliferation and monocyte infiltration, a blockade of TNF-α, and an inhibition of the COX pathway (Pahwa et al., 2020). This pathway of a mode of action in inflammation may have been followed by this leaf extract.

The identified phytoconstituents in *M. sativa* have a documented mechanism of action against inflammation in the literature. One way to achieve this is by suppressing the production of inflammatory enzyme indicators like COX-2 and COX-5, and also by inhibiting the activation of NF-KB (Firke and Bari, 2015). In all three test phases, the development of carrageenan paw-induced edema was significantly decreased by *M. sativa* leaf extract. Different mediators may operate to trigger an inflammatory response, which could lead to the edema formation (Necas and Bartosikov, 2013). These authors claim that prostaglandins are only visible during the last stages of inflammation, but bradykinin was one of the early mediators. Acetylsalicylate acid (aspirin) and other nonsteroidal anti-inflammatory drugs may not prevent the first stage of edema caused by carrageenan, but they may counteract the second, faster phase of the edema (Mallinson, 2017).

Local neutrophil infiltrations aided in the inflammatory response, producing mediators such as hydroxyl radicals and superoxide anion (O^ (2-) (Girard et al., 2016). The diminution of swelling observed in the carrageenan-induced paw was linked to the induced lowering of COX-2 (Xu et al., 2012). The results of this study's carrageenan-induced paw edema test on the effects of the *Medicago sativa* extract indicated that it may have produced anti-inflammatory activity by inhibiting prostaglandin synthesis and reducing inducible cycloxygenase, NO, and other cytokines. However, to confirm this potential impact, specific mechanism(s) of action experiments must be carried out. This study showed that carrageenan-induced subplanter edema in Wistar rats was alleviated by *M. sativa* leaf extract in ethanol. Rat paw edema induced by carrageenan was a helpful test for assessing the efficacy of anti-inflammatory agents which works by blocking the mediators of acute inflammation (Rahman and Jahan, 2021).

One significant process brought on by an inflammatory stimulation is the disruption of the neutrophil membrane. Superoxide and other extremely reactive oxygen species are typically produced by this. According to Chang et al., (2017), the extract's action appears to correspond with the release of serotonin and cytoplasmic enzymes by mast cells during the nonphagocytic phase of inflammation caused by carrageenan. Anele et al., (2024) conducted a study on the phytochemical composition of *M. sativa*, which revealed the presence of alkaloids, flavonoids, and saponins. Additionally, the study demonstrated a greater level of polyphenols content, which includes flavonoids, phenol, and tannins. Given that other studies have shown that the bioactive phytoconstituents alkaloids, flavonoids, phenols, and tannins have potent analgesic effects, it is possible that these compounds may account for *M. sativa's* analgesic properties.

4. CONCLUSION

According to the current study's findings, *Medicago sativa* leaf extract extracted with ethanol has potent analgesic and anti-inflammatory properties in a mouse model similar to those of the prescription medication acetylsalicylic acid. The findings of this study are consistent with previous studies on the analgesic and anti-inflammatory effects of plant extracts. However, there is need for more investigation to clarify the precise mechanisms behind the benefits seen. The research opens up new possibilities for future studies and the creation of innovative painkiller drugs by providing insightful information about the possible therapeutic application of *M. sativa* in pain management.

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Authorship contribution statement

Anele DO & Akuodor GC conducted the research. Ofonakara U & Nwokike MO conducted the literature search. Ajegi IF & Igwe DO conducted data analysis. Austine-Abu JU, Asogwa RE & Ofor CC wrote and prepared the manuscript. All authors approved the final version of the manuscript.

Ethical approval

The Animal ethical guidelines are followed in the study for experimentation. Experimental procedures were performed in accordance with the Ethics Committee of the National Research Centre and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996). As per the plant ethical regulations & specimen ethical regulations, the sample specimen for *Medicago sativa*, MOUAU/ZEB/21/009 was placed in the university herbarium for reference after the plant was recognized and verified by Mr. Ibe Ndukwe, a taxonomist at the Department of Forestry, College of Environmental Sciences, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

Informed consent

Not applicable

Conflicts of interests
The authors declare that there are no conflicts of interests.

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Data and materials availability
All data associated with this study are present in the paper.

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