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Evaluation of the Antitussive and Analgesic Activities of *Peperomia pellucida* Whole Plant Methanol Extract in Rodents

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ABSTRACT

Background and Purpose: *Peperomia pellucida* (Piperaceae) is a plant used in ethnomedicine to treat asthma, colds, coughs, and other illnesses. This study aimed to evaluate the antitussive, expectorant, and analgesic activities of a 70% methanol extract of the plant.
Methods: Citric acid and ammonia induced rodent cough models; and phenol red dye expectorant model in mice were used to evaluate the antitussive and expectorant properties of the orally administered extract. The hot plate and acetic acid induced mouse writhing models were used to evaluate analgesia.
Results: Dihydrocodeine (25 mg/kg); and 100, 200, and 400 mg/kg of the extract significantly reduced the frequency of cough bouts and increased the percentage suppression of cough in citric acid and ammonium hydroxide induced cough models compared with control ($P<0.05$, $P<0.01$, $P<0.001$). Bromhexine (15 mg/kg); 100 and 400 mg/kg of the extract increased the secretion of phenol red dye in mice when compared with control ($P<0.05$, $P<0.01$). Doses of the extract (100, 200 mg/kg) and pentazocine (3 mg/kg) increased ($P<0.05$) the latency to response time in hot plate induced pain. At 400 mg/kg, the extract; and acetylsalicylic acid (100 mg/kg) reduced the number of writhing and increased the percentage inhibition of pain in the acetic acid induced model when compared to control ($P<0.05$, $P<0.001$).
Conclusion: The 70% methanol extract of *P. pellucida* possesses antitussive, expectorant, and analgesic activities in rodents.

INTRODUCTION

In Nigeria, medicinal plants are the main sources of medications used in both traditional and modern medicine as dietary supplements and pharmaceuticals. More than 70% of contemporary pharmacological products are estimated to be derived from herbs (Brisibe *et al*., 2008). Since medicinal plants contain significant chemical components that can stimulate specific physiological effects in both humans and animals, they have for long been utilized to treat diseases. The organic substances are known as phytochemicals (Balunas and Kinghorn, 2005; Sen *et al*., 2010; Paul *et al*., 2015; Koche *et al*., 2016) and they prevent illness and promote wellness (Narasinga, 2003).

Peperomia pellucida is an annual herbaceous plant (Cao, 2012) belonging to the family Piperaceae (Ghani, 1998). It is also known as clearweed, pepper elder, silver bush, shiny bush and in Nigeria, the Yorubas call it "rinrin" (Amarathunga and Kankanamge, 2017; Usman and Ismaeel, 2020). Ethnobotanical studies indicate that the plant has long been used medicinally for its CNS, analgesic, antipyretic, anti-inflammatory, and antibacterial properties (Majumder and Kumar, 2011). Asthma, vaginal infections, kidney infections, viral infections, fever, colds, coughs, rheumatic pain, and many more illnesses are treated with it (Ragasa, 1998; Khan and Omoloso, 2002). The aerial parts are used as bandages for wounds, while the roots are utilized to treat fevers (Hartati *et al*., 2015). The main chemical constituents present in *P. pellucida* are alkaloids, flavonoids, sterols, tannins, reducing sugars, saponins, trieterpenoids, carbohydrates, phenols, azulenes, carotenoids, depsides and quinones (Alves *et al*., 2019). This study was aimed at evaluating the antitussive, expectorant, and analgesic activities of 70% methanol extract of *P. pellucida* plant.

MATERIALS AND METHODS

Collection of plant materials

Fresh whole plants of *Peperomia pellucida* were collected between November and December, 2019 from a farmland in Ogbeson in Ikpoba-Okha Local Government Area, Edo State, Nigeria. The plant was identified and authenticated by Dr. H. Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria with a voucher number UBH-P602.

Preparation of 70 % methanol extract

Ninety-five grams (95 g) of the whole plant was washed with distilled water and ground with 300 mL of distilled water using an electric blender. The ground material was then mixed with 700 mL of methanol in an airtight bottle for 72 h, with frequent shaking. The mixture was filtered using a cheesecloth, and the filtrate obtained was filtered again using Whatman filter paper grade 1(Cat No. 1001 125). The filtrate was concentrated over a water bath and dried in an oven at 40℃ and the extract was placed in an air-tight container and stored in a refrigerator.

Experimental animals

Mice weighing 25-30 g and guinea pigs weighing 300-500 g were purchased from the animal farm of the College of Medicine, Ambrose Alli University, Ekpoma, Edo State, Nigeria. They were housed in the animal house of the Department of Animal and Environmental Biology (AEB), Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria. The animals were allowed two weeks of acclimatization and housed under natural laboratory conditions with a 12 h light/dark cycle. They were fed with standard growers' pellets and allowed unlimited access to potable water. All the guidelines of the National Institute of Health USA: Public Health Service Policy on humane care and use of laboratory animals (2002) were complied with.

Experimental Design for Antitussive and Expectorant Activities

Citric acid induced cough in guinea pigs

Citric acid induced cough in guinea pigs was evaluated using the method of Nadig and Laxim (2005), modified by Adejayan *et al*. (2019). A day before the test, 35 guinea pigs were placed individually in a chamber (24×12×24 cm) and exposed to citric acid aerosol (7.5% w/v) using an Omron® (Omron Health Care Ltd., Japan) compressor nebulizer (rate of 0.3 mL/min and particle size 3 µm) for 5 min. The animals that gave 10 cough bouts or more were selected for the study and fasted overnight. Twenty-five guinea pigs that gave 10 cough bouts and above were randomly allocated into five groups (n=5) as follows: Group 1 served as control and was given 5 mL/kg of distilled water; Group 2 was given 100 mg/kg of the extract; Group 3 was given 200 mg/kg of the extract; Group 4 was given 400 mg/kg of the extract; and Group 5 was given 25 mg/kg of dihydrocodeine (DHC). The standard drug, the extract and distilled water were given orally using an orogastric tube. After an hour of administration, the animals were re-exposed to citric acid aerosol as described above and the numbers of cough bouts were recorded. The percentage of cough suppression was calculated using the formula:

$$\frac{No.of\ Pretreatment\ Bouts - No.of\ Post\ Treatment\ Bouts}{No.of\ Pretreatment\ Bouts} \times 100$$

Ammonia induced cough in mice

The ammonium hydroxide induced cough in mice was evaluated using the method described by Adejayan *et al*. (2019). Twenty-five mice were randomly allocated into five groups (n=5), as follows: Group 1 was given 2 mL/kg of distilled water as a control; Group 2 was given 100 mg/kg of the extract; Group 3 was given 200 mg/kg of the extract; Group 4 was given 400 mg/kg of the extract; and Group 5 was given 25 mg/kg of dihydrocodeine. All treatments were

by use of orogastric tube. One hour after administration, each mouse was placed in 1 L (10×10×10 cm) chamber containing 0.3 mL of 25% ammonium hydroxide in a cotton wool for 45 s. The number of cough bouts for each mouse was recorded after 5 min of exposure and the percentage inhibition was calculated thus:

$$\frac{\text{Mean Bouts (Control)} - \text{Mean Bouts (Treated)}}{\text{Mean Bouts (Control)}} \times 100$$

Mucus expectorant (phenol red dye secretion) in mice

Mucus expectoration in mice was examined using a method described by Adejayan *et al*. (2019). Mice (30) were randomly allocated into six groups (n=5) as follows: Group 1 was given 2 mL/kg of distilled water daily for 5 days as the control; Group 2 was given 100 mg/kg of the extract daily for 5 days; Group 3 was given 200 mg/kg of the extract daily for 5 days; Group 4 was given 400 mg/kg of the extract daily for 5 days; Group 5 was given 15 mg/kg of bromhexine hydrochloride daily for 5 days; and Group 6 was given 50 mg/kg of sodium cromoglycate at day five. All treatments were given orally using an orogastric tube, except sodium cromoglycate, which was given intraperitoneally. Thirty minutes after the administration of extract, bromhexine hydrochloride and sodium cromoglycate, a secretagogue, ammonium chloride (5 mg/kg *p.o.*) was administered. Another 30 min later, each mouse was injected with phenol red (500 mg/kg *i.p.*). All the mice were sacrificed by cervical dislocation 30 min after phenol red injection. A piece (2 cm) of the trachea from the thyroid cartilage to the main stem bronchi was isolated. Each piece of tracheae was stored for 30 min in 2 mL normal saline. A volume of 0.1 mL of 1 M sodium hydroxide was added to the fluid to stabilize the pH. The absorbance of phenol red released from each piece of tracheae was read at 460 nm using a T80+UV/VIS spectrophotometer (PG Instruments Ltd, Beijing, China). A standard curve (graph of absorbance against concentration, R^2 = 0.987) was plotted from which the concentration of phenol red was extrapolated.

Experimental Design for Analgesic Activity

Hot plate induced pain in mice

The hot plate induced pain in mice was examined using a method described by Olayinka *et al*. (2021). Mice (25) of either sex were randomly allocated to five groups (n=5). The first group received 2 mL/kg of distilled water; the second group received 100 mg/kg of the extract; the third group received 200 mg/kg of the extract; the fourth group received 400 mg/kg of the extract; and the fifth group received pentazocine (3 mg/kg). All treatments were given orally using an orogastric tube, except pentazocine, which was given intraperitoneally. The hot plate was turned on and the temperature was maintained at 55 ± 0.5ºC. A cut-off time of 30 s was adopted to avoid burning or tissue damage. Hind paw licking or biting, and jumping were interpreted as pain perception signals. At 30 min after oral administration, each mouse was positioned on the hot plate and response latency (time between placement and licking or biting the hind paws, or jumping) was recorded at time intervals of 60, 120, 180, and 240 min.

Acetic acid induced writhing in mice

Acetic acid induced writhing in mice was examined using the method described by Koster *et al*. (1959) and modified by Olayinka *et al*. (2021). Mice (25) of either sex were randomly allocated to five groups (n=5): Group 1 was given 2 mL/kg of distilled water as a control; Group 2 was given 100 mg/kg of the extract; Group 3 was given 200 mg/kg of the extract; Group 4 was given 400 mg/kg of the extract; and Group 5 was given acetylsalicylic acid (100 mg/kg). All treatments were given orally using an orogastric tube. Thirty minutes later, pain stimulus was induced by intraperitoneal injection of 10 mL/kg of 0.6% solution of acetic acid into every mouse in each group. Mice were placed in individual observational cages and the total numbers of writhing (which is characterized by elongation of the body and the development of tension in the abdominal muscles and hind paws) were counted for 30 min following acetic acid injection. Percentage reduction in writhing (an index of pain inhibition) was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Wc- Wt}}{\text{Wc}} \times 100$$

Where Wt = mean writhing of treatment group; Wc = mean writhing of control group.

Statistical Analysis

Results are presented as mean ± standard error of mean (SEM) and 'n' represents the number of guinea pigs or mice per experimental group. Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Data were analyzed using GraphPad Prism (UK) software version 6. $P<0.05$ shows significance difference between compared data.

RESULTS

Effect of extract on citric acid induced cough

Table 1 shows the suppressive effect of the 70% methanol extract of *P. pellucida* on citric acid induced cough in guinea pigs. Dihydrocodeine (25 mg/kg) ($P<0.001$), and 100 mg/kg ($P<0.05$), 200 mg/kg ($P<0.05$), 400 mg/kg ($P<0.01$) of the extract significantly reduced the frequency of cough bouts and increased the percentage suppression of cough compared with control.

Effect of extract on ammonium hydroxide induced cough in mice

Figure 1 shows the effect of the extract on ammonium hydroxide induced cough in mice. The extract at 100 ($P<0.05$), 200 ($P<0.05$) and 400 mg/kg ($P<0.05$); and 25

mg/kg of dihydrocodeine (DHC) (P<0.01) significantly reduced the number of cough bouts compared with control.

Effect of extract on mucus expectoration in mice

Figure 3 shows the effect of the extract of *P. pellucida*, bromhexine (BH), and sodium cromoglycate (NaCr) on phenol red dye secretion in mice. Bromhexine (15 mg/kg) (P<0.01), 100 (P<0.01) and 400 mg/kg (P<0.05) of the extract significantly increased the secretion of phenol red dye in mice when compared to control.

Hot plate induced pain in mice

Table 2 shows the effect of 70 % methanol extract of *P. pellucida* on hot plate induced pain in mice. The extract at doses of 100, 200 mg/kg, and pentazocine (3 mg/kg) significantly (P<0.05) increased response latency in hot plate induced pain in mice.

Acetic acid induced writhing in mice

Table 3 shows the effect of the extract of *P. pellucida* on acetic acid induced writhing in mice. The extract at the dose of 400 mg/kg (P<0.05), and 100 mg/kg of acetylsalicylic acid (P<0.001) significantly reduced the number of writhing and increased the percentage inhibition of pain.

Table 1: Suppressive effect of a 70 % methanol extract of *P. pellucida* on citric acid induced cough in guinea pig.

Groups	Treatment	Dose	Frequency of Cough Bout (Before)	Frequency of Cough Bout (After)	Suppression (%)
I	Control	10 ml/kg	15.25 ± 2.78	12.75±3.01	18.78±4.80
II	Extract	100 mg/kg	17.25±3.86	6.25±0.63*	60.10±6.90*
III	Extract	200 mg/kg	12.75±2.14	5.75±0.65*	52.85±7.61*
IV	Extract	400 mg/kg	13.75±1.32	4.00±0.71**	69.23±7.53**
V	Dihydrocodeine	25 mg/kg	18.75±4.19	2.75±0.48***	67.50±17.32*

*P<0.05, **P<0.01, ***P<0.001 versus control. Values are presented as mean ± S.E.M, n=5

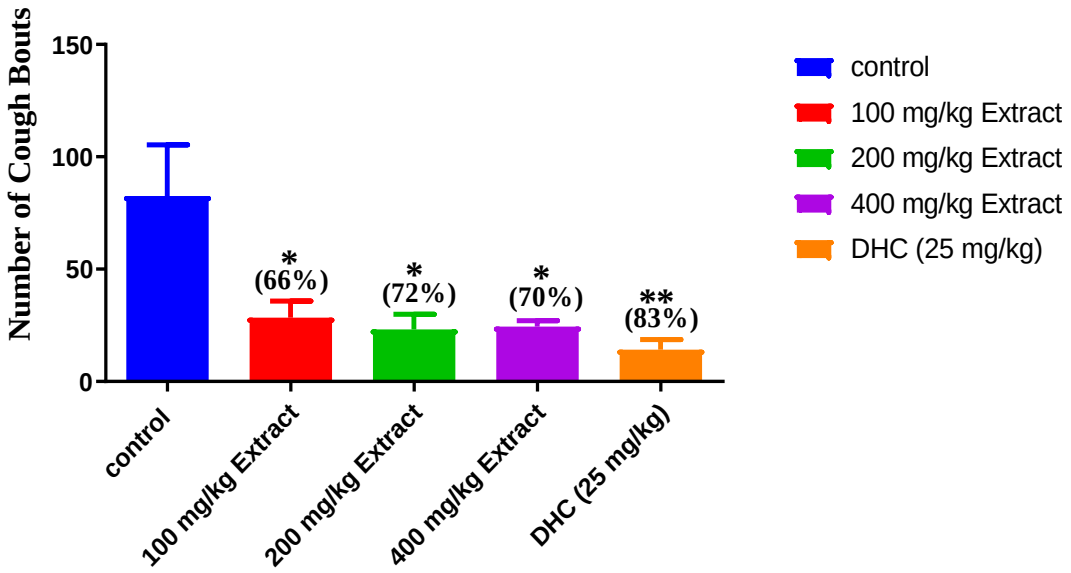

Figure 1: The effect of administration of 70 % methanol extract of *P. pellucida* on ammonium hydroxide induced cough in mice. DHC: dihydrocodeine. *P<0.05, **P<0.01 versus control. Values are presented as mean ± SEM, n=5.

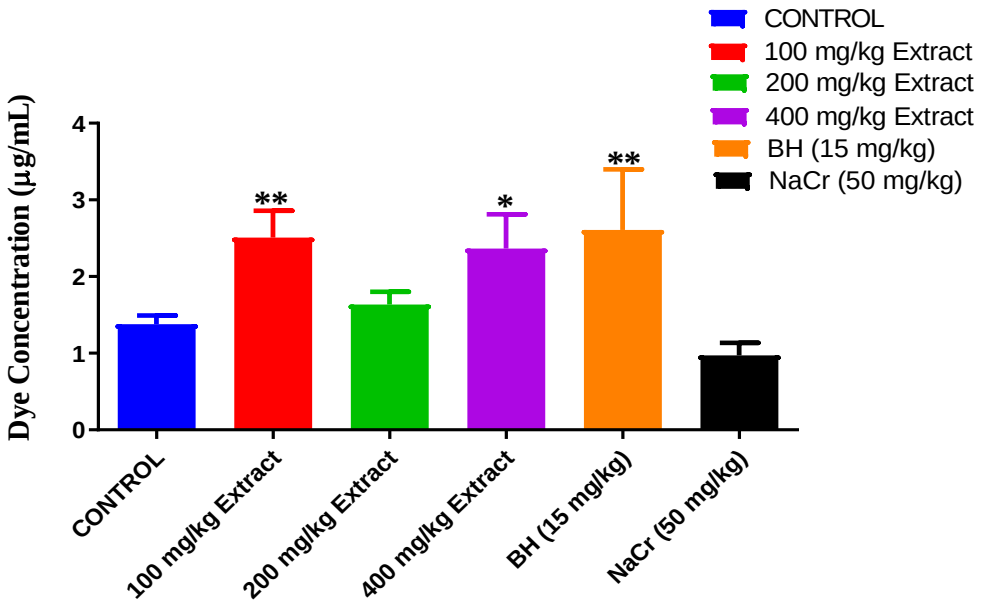

Figure 2: The effect of 70 % methanol extract of *P. pellucida*, bromhexine (BH), and sodium cromoglycate (NaCr) on phenol red dye secretion in mice. **P*<0.05, ***P*<0.01 versus control. Values are represented as Mean ± S.E.M, n=5.

Table 2: The effect of 70 % methanol extract of *P. pellucida* on hot plate induced pain in mice.

	Latency Time (s)			
Groups	**60 min**	**120 min**	**180 min**	**240 min**
Control (10 ml/kg)	7.40±1.17	7.80±0.20	8.40±0.40	12.00±1.41
100 mg.kg (Extract)	9.20±1.16	8.20± 0.58	14.20±0.37λ	14.20±2.65
200 mg/kg (Extract)	8.40±1.63	15.60±1.57$^{\lambda}$	11.40±0.51*****	13.60±2.02
400 mg/kg (Extract)	5.20±0.20	8.60±1.03	8.80±1.11	12.40±0.25
3 mg/kg (Pentazocine)	8.60±1.72	12.20±0.49*****	11.60±0.51*****	12.20±1.69

**P*<0.05 versus control. Values are presented as mean ± S.E.M. where number of replicates, n=5 per group.

Table 3: The effect of 70 % methanol extract of *P. pellucida* on acetic acid induced pain in mice.

Groups	**Number of Writhing**	**% Inhibition**
Control (10 ml/kg)	129.00±10.31	0
100 mg/kg (Extract)	111.00±18.90	14
200 mg/kg (Extract)	124.20±5.72	4
400 mg/kg (Extract)	76.16±7.30*****	41
100 mg/kg (ASA)	52.00±5.94*******	60

**P*<0.05, ****P*<0.001 versus control. Values are presented as mean ± S.E.M, n=5 per group, ASA= acetylsalicylic acid.

DISCUSSION

The antitussive activity of the 70% methanol extract of *Peperomia pellucida* was carried out using citric acid induced cough, ammonium hydroxide induced cough, and phenol red expectoration models. Cough can be elicited in an experimental animal by mechanical, electrical, and chemical stimulation. Chemical stimulation is the most popular experimental model because it is like a physiological event (Adejayan *et al*., 2019). Results from this study show that 25 mg/kg dihydrocodeine; 100, 200, and 400 mg/kg of the extract significantly reduced the frequency of cough bouts and increased the percentage cough suppression. Citric acid acts as a tussigenic agent, which when inhaled stimulates transient receptors on the C sensory fibres, causing the release of tachykinins, which stimulate bronchoconstriction and mucus secretion (Helms *et al*., 2008). The results from this work also show that the extract at all dose levels (100, 200, 400 mg/kg) and dihydrocodeine (25 mg/kg) significantly reduced the number of cough bouts in the ammonium hydroxide model. Lin *et al*. (2008) suggest that ammonium hydroxide irritates the bronchial mucosa wall, leading to secretion of excess fluid in the tracheobronchial airways for easier clearance of mucus (Lin *et al*., 2008). The results showed that the extract of *P. pellucida* has the ability to reduce the frequency of cough bouts and increase the percentage suppression of cough. Dihydrocodeine was used as the standard drug because it is the second most specific antitussive opioid that is commonly used (Eddy *et al*., 1969). It acts on the μ opioid receptors to suppress the cough reflex (Adejayan *et al*., 2019). Thus, the mechanism of action of *P. pellucida* may be due to its stimulant action on μ opioids receptors leading to the suppression of the cough reflex. Guinea pigs were used for citric acid induced cough because their airways possess the needed afferent nerves and they can produce cough just like humans (Adejayan *et al*., 2019).

The expectorant activity of the extract was determined using a trachea phenol red secretion assay. This study shows that the extract increased the secretion of phenol red dye in the mouse trachea. This model is based on the principle that when phenol red is given intraperitoneally with an expectorant drug (which increases secretion of mucus in the trachea in order to clear the airways), there would be a discharge along with the tracheal secretions. Bromhexine (15 mg/kg) was used as the standard drug because it is a mucolytic drug that enhances secretion and dilution of the phlegm in the respiratory tract, thus increasing the excretion of phenol red (Zhou *et al*., 2013). The basis of using expectorants as cough relieving agents rests in the possibility that alteration of the volume of secretions or their composition may lead to suppression of cough reflexes (Chung, 2008). The increases in secretion of phenol red dye in mice by 100, 400 mg/kg of the extract; and bromhexine, were comparable. Thus, the expectorant activity of the extract could be attributed to its ability to increase phlegm secretion and dilution in the respiratory tract. This study has therefore established the basis for the use of *P. pellucida* as an antitussive in ethnomedicine.

The analgesic activity of *P. pellucida* was determined using the hot plate test and an acetic acid induced writhing test in mice. Pain can be defined as some unfriendly sensory and emotional experience linked with actual or potential tissue injury or described in terms of such injury (Raja *et al*., 2020; Yam *et al*., 2018). Hence, pain is a specific sensation with its own peripheral and central mechanisms independent of the other five senses. Chemical mediators responsible for pain activation include histamine, substance P, bradykinin, acetylcholine, leukotrienes, and prostaglandins all of which are important in the treatment of pain (Apkarian *et al*., 2005). This study found that extract of *P. pellucida* (100, 200 mg/kg) and pentazocine (3 mg/kg) significantly increased response latency in the hot plate induced pain in mice. Pentazocine was used as the standard drug because it acts centrally at the μ opioid receptors to inhibit pain reflexes. The results in this study show that an increase in response latency by the extract of *P. pellucida* and pentazocine were comparable (Table 2). Thus, the analgesic activity of *P. pellucida* may be due to its central action on μ opoids receptors.

The analgesic activity was further evaluated using acetic acid to induce pain in mice. The acetic acid model is a sensitive procedure to evaluate peripherally acting analgesics. Acetic acid causes pain by liberating endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins, and substance P (Shivaji, 2012; Bentley *et al*. 1983). It has been noted that the level of analgesia in the acetic acid-induced model is related to the percentage reduction in the number of abdominal constrictions or writhing (Machioro *et al*., 2005). The extract at 400 mg/kg and aspirin (100 mg/kg) reduced the number of writhing and also caused an increase in the percentage pain inhibition. Aspirin was used as the standard drug because it is a non-steroidal anti-inflammatory drug (NSAID) that inhibits the activity of cyclooxygenase, thereby preventing the synthesis of prostaglandins that cause inflammation, swelling, pain, and fever (Zimmermann and Curtis, 2018). Thus, the analgesic activity can be likened to that of a peripherally-acting NSAID. The analgesic activity of *P. pellucida* may be centrally and peripherally acting since there was significant activity recorded in both methods (hot plate and acetic acid) used.

CONCLUSION

Peperomia pellucida possesses antitussive, expectorant, and analgesic activities. Its analgesic effect may be centrally and/or peripherally mediated. The results lend credence to its ethnomedicinal uses for the treatment of cough and pain. However, further study will need to thoroughly investigate the mechanisms of action for cough and pain.

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CONFLICT OF INTEREST

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AUTHORS CONTRIBUTIONS

DOU designed the study; DOU, FOI and ONA carried out the experiments. FOI analysed the data and wrote the manuscript. The entire process was supervised by DOU. All authors read the manuscript and approved its submission.

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