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Anti-Nociceptive Effects and the Mechanism of *Palisota hirsuta* K. Schum. Leaf Extract in Murine Models

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Abstract: The anti-nociceptive effect of an ethanolic leaf extract of *Palisota hirsuta*, a plant used locally in Ghana for various painful conditions was assessed, using various pain models. *Palisota hirsuta* extract (PHE) together with morphine and diclofenac (positive controls), all showed significant dose-dependent anti-nociceptive activity in all the models used, that is the tail withdrawal test, the inflammatory-induced mechanical hyperalgesia test, the acetic acid induced writhing test and the formalin test. The anti-nociceptive effect exhibited by PHE in the formalin test was reversed by the systemic administration of the non-selective opioid antagonist, naloxone, the NO synthase inhibitor, N_{ω}-nitro-arginine methyl ester (L-NAME) and the ATP-sensitive K^+ channel inhibitor, glibenclamide. However, theophyline, a non-selective adenosine receptor antagonist did not reverse this effect. PHE, unlike morphine, did not induce tolerance to its anti-nociceptive effect in the formalin test after chronic administration and also morphine tolerance did not cross-generalize to PHE. Overall, the present results demonstrate that the anti-nociceptive effects of PHE might partially or wholly be due to the stimulation of peripheral opioid receptors through the activation of the nitric oxide-cyclic GMP-ATP-sensitive K^+ (NO/cGMP/K^+ATP)-channel pathway without tolerance induction after chronic administration.

Key words: *Palisota hirsuta*, morphine, diclofenac, naloxone, L-NAME, glibenclamide

INTRODUCTION

Palisota hirsuta K. Schum. (Family: Commelinaceae), known locally in Ghana as *somenini* or *mpentemi* (Twi), *sombenyin* (Fante) and *sumbe* (Ewe). It is a robust herb found in forest regrowths and is about 2-4 m high. In Ghana, this plant and several others are used either alone or as combination therapy with orthodox medicine in the treatment of various painful conditions.

The whole plant and various parts are used extensively in West African medicine for various conditions. In Ghana, the whole plant is used for stomach pains and the sap from the roasted leaves is instilled in the ear for earache whilst heated leaves are applied over the lumbar region for kidney pains (Burkill, 1985). Also, a leaf infusion or poultice is taken orally or applied locally for piles (Dokosi, 1998; Burkill, 1985). The dried leaves are smoked for toothache (Burkill, 1985; Dokosi, 1998). The Igbo of Obompa in Nigeria prepare an ointment made from this plant for gunshot wounds and swellings (Burkill, 1985).

Apart from the anti-viral work done by Anani and co-workers (Anani *et al*., 2000; Hudson *et al*., 2000), not much has been reported on this plant. The present study reports on the anti-nociceptive effects of the ethanolic extract of the leaves in animal models of analgesia and its possible mechanism of action.

MATERIALS AND METHODS

Plant material: Leaves of the plant *Palisota hirsuta* were collected from the Botanic Gardens of Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, between January and February, 2007. The leaves were authenticated by Mr. Amissah, the curator of the garden and a voucher specimen (No. FP 10081) has been kept in the Faculty of Pharmacy Herbarium, KNUST, Kumasi.

Preparation of extract: The leaves were air-dried indoors for a week and pulverized with a hammer-mill. The powder was extracted by cold maceration using 70% (v/v) ethanol over a period of 72 h. The resulting filtrate was concentrated under low temperature (60°C) and pressure to a syrupy mass in a rotary evaporator. The syrupy mass was then dried to a dark brown semi-solid mass using water bath and kept in a dessicator till it was ready

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to be used. The final yield was 10.5% (w/w). This is subsequently referred to as PHE or extract.

Drugs: Diclofenac sodium was purchased from Troge, Hamburg, Germany, morphine hydrochloride from Phyto-Riker, Accra, Ghana, carrageenan sodium salt, naloxone hydrochloride and N_{ω}-Nitro-l-arginine methyl ester (L-NAME) were also obtained from Sigma-Aldrich Inc., St. Louis, MO, USA, formalin, acetic acid and theophyline were also purchased from BDH, Poole, England whilst glibenclamide, Daonil® was from Sanofi-Aventis, Guildford, UK).

Animals: Sprague-Dawley rats (150-200 g) and ICR mice (20-25 g) of both sexes were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and housed in the animal facility of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST). The animals were housed in groups of six in stainless steel cages (34×47×18 cm^3) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions (temperature 24-28°C, relative humidity 60-70% and 12 h light-dark cycle). All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication No. 85-23, revised 1985). All protocols used were approved by the Departmental Ethics Committee.

Phytochemistry: The presence of saponins, alkaloids, triterpenes, flavonoids, glycosides, reducing sugars and tannins were tested by simple qualitative and quantitative methods of Trease and Evans (1989).

Tail immersion test: The tail immersion test was carried out according to the method described by Janssen *et al.* (1963) and modified by Savegnago *et al.* (2007). Tail withdrawal latency, defined by the time (in seconds) to withdraw the tail from hot water maintained at 50.0±1.0°C, was measured using a stopwatch. A cut-off time of 10 sec was set to avoid tissue damage. Increase in tail withdrawal latency was defined as anti-nociception and calculated as % maximum possible effect (MPE). The maximum possible ant-inociceptive effect was reached when the animals did not show a tail withdrawal reaction within the cut-off time of 10 sec. % MPE was calculated according to the formula: $[(T_1-T_0)/(T_2-T_0)]\times100$, where T_0 and T_1 are the latencies obtained before and after drug treatment and T_2 is the cut-off time. Rats were tested before and at 30, 60, 90, 120, 150 and 180 min after administration of PHE (30-300 mg kg^{-1}, p.o.), morphine (1-10 mg kg^{-1}, i.p.) or diclofenac (10-100 mg kg^{-1}, i.p.). A single habituation test was used before baseline test to minimize learning effects.

Carrageenan-induced mechanical hyperalgesia: Mechanical nociceptive thresholds were measured in the rat paw pressure test (Randall and Selitto, 1957) and as modified by Villetti *et al.* (2003) and Stöhr *et al.* (2006), using an analgesimeter (Model No.15776, Ugo Basile, Comerio, Varese, Italy) which is based on the Randall-Selitto test (Randall and Sellito, 1957). This was used to apply a linearly-increasing pressure, by means of a blunt perspex cone, to the dorsal region of the right hind paw until the rat withdrew the paw. Rats received two training seasons before the day of testing. Pressure was gradually applied to the right hind paw and paw withdrawal thresholds (PWTs) were assessed as the pressure (grams) required to elicit paw withdrawal. A cut-off point of 250 g was used to prevent any tissue damage to the paw. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect (% MPE). On the test day, a baseline measurement was taken before animals were administered carrageenan (100 μL of a 20 mg mL^{-1} solution) into the right hind paw. PWTs were determined again 2.5 h after carrageenan to establish that mechanical hyperalgesia had developed. PHE (30-300 mg kg^{-1}, p.o.), morphine (1-10 mg kg^{-1}, i.p.) or diclofenac (10-100 mg kg^{-1}, i.p.) were then administered 3 h post-carrageenan and PWTs were taken again at 3.5, 4, 4.5, 5, 5.5 and 6 h post-carrageenan.

Acetic acid-induced abdominal constriction: ICR mice (20-30 g) were used according to the method described by Amresh *et al.* (2007) with slight modifications. The total number of writhings following intraperitoneal administration of 10 mL kg^{-1} of 0.6% acetic acid was recorded over a period of 20 min, starting 10 min after the acetic acid injection. The response induced by i.p. injection of acetic acid consists of a contraction of the abdominal muscle, together with a stretching of the hind limbs. Animals received PHE (30-300 mg kg^{-1}, p.o.), diclofenac (10-100 mg kg^{-1}, i.p.) or morphine (1-10 mg kg^{-1}, i.p.) 30 min before the acetic acid administration. Control animals received vehicle.

Formalin induced nociception: The formalin test first described by Dubuisson and Dennis (1977) was carried out as described by Malmberg and Yaksh (1995) with a few modifications. Each animal was assigned and acclimatized to one of 20 formalin test chambers (a perspex

chamber 15×15×15 cm^3) for 30 min before formalin injection (Wilson *et al*., 2002). The mice were then pre-treated with the test drugs (30 min for i.p. route and 1 h for oral route) before intraplantar injection of 10 μL of 5% formalin. The animals were immediately returned individually into the testing chamber. A mirror angled at 45° below the floor of the chamber allowed a complete view of the paws. The behaviour of the animal was then captured (60 min) for analysis by a camcorder (Everio™ model GZ-MG1300, JVC, Tokyo, Japan) placed in front of the mirror. Pain response was scored for 60 min, starting immediately after formalin injection.

The first phase of the formalin test was defined conservatively as 0-10 min and the second phase 10-60 min post formalin injection (Wilson *et al*., 2002). Nociceptive behaviour was quantified by counting the incidents of spontaneous biting/licking of the injected paw (Hayashida *et al*., 2003) using the public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/). Nociceptive score was determined for each 5 min time block in each phase by measuring the amount of time spent biting/licking the injected paw. The product of the frequency and duration of biting/licking was used as nociceptive score. Mice were randomly selected for one of the following study groups:

Group I: Morphine (1, 3 and 10 mg kg^{-1})
Group II: *Palisota* extract (30, 100 and 300 mg kg^{-1})
Group III: Vehicle treated control

Extract was prepared in 2% tragacanth mucilage. Drug solutions and suspensions were prepared such that not more than 1 mL of extract was given orally and not more than 0.5 mL of the standard drugs were injected intraperitoneally. All drugs were freshly prepared.

Analysis of the mechanism of action of PHE in the formalin test: To investigate the possible mechanisms by which PHE inhibits formalin-induced nociception, mice were pre-treated with different drugs. The doses of antagonist, agonist and other drugs were selected on the basis of earlier literature data and in pilot experiments in our laboratory. The formalin test was chosen for this purpose because of the specificity and sensitivity in nociception transmission that this model provides (Le Bars *et al*., 2001).

Involvement of opioid system: To assess the participation of the opioid system, mice were pre-treated intraperitoneally (i.p.) with naloxone (2 mg kg^{-1}, a non-selective opioid receptor antagonist). After 15 min the animals received PHE (100 mg kg^{-1}, p.o.), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL kg^{-1}, p.o.). The nociceptive response to the formalin intraplantar injection was recorded 60 min after administration of PHE or vehicle and 30 min after administration of morphine.

Involvement of adenosinergic system: To investigate the role played by the adenosinergic systems in the anti-nociception caused by PHE, mice were pre-treated with theophylline (5 mg kg^{-1}, i.p., a non-selective adenosine receptor antagonist). After 15 min, the mice received PHE (100 mg kg^{-1}, p.o.), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 mL kg^{-1}, p.o.). The nociceptive response to the formalin intraplantar injection was recorded 60 min after administration of PHE or vehicle and 30 min after morphine administration.

Involvement of ATP sensitive K^+ channels: To explore the possible contribution of ATP sensitive K^+ channel in the anti-nociceptive effect of PHE, mice were pre-treated with glibenclamide (an ATP-sensitive K^+ channel inhibitor, 8 mg kg^{-1}, p.o.), or vehicle and after 30 min they received PHE (100 mg kg^{-1}, p.o.), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive responses to formalin were recorded 60 min after administration of PHE or vehicle and 30 min after morphine administration.

Participation of the nitric oxide system: To verify the possible involvement of nitric oxide/cyclic GMP pathway in the anti-nociceptive action caused by PHE, mice were pre-treated with N_ω-L-nitro-arginine methyl ester (L-NAME, a NO synthase inhibitor; 10 mg kg^{-1}, i.p.) or saline (0.9% NaCl, i.p.) 30 min before PHE (100 mg kg^{-1}, p.o.), morphine (3 mg kg^{-1}, i.p.) or vehicle administration. The nociceptive responses to formalin were recorded 60 min after administration of PHE or vehicle and 30 min after morphine administration.

Tolerance studies: The mouse paw formalin test was used to ascertain whether, after chronic treatment, tolerance develops to the anti-nociceptive activity of PHE and morphine. Mice were divided randomly into five groups (n = 5) and treated once daily for 8 days as follows: three groups with saline i.p., one group with PHE 200 mg kg^{-1}, p.o. and one group with morphine 6 mg kg^{-1}, i.p. On day 9, these groups were treated in the following manner: one saline-pre-treated group was treated with saline i.p.; two saline-pre-treated groups were treated either with PHE

100 mg kg^{-1}, p.o. or with morphine 3 mg kg^{-1}, i.p.; the group pre-treated with PHE 200 mg kg^{-1} was treated with PHE 100 mg kg^{-1}, p.o. and the group pre-treated with morphine 6 mg kg^{-1} was treated with morphine 3 mg kg^{-1}, i.p. PHE and morphine were administered 60 and 30 min before formalin injection, respectively. In a separate study, PHE was administered to animals chronically treated with morphine to establish whether morphine-induced tolerance cross-generalizes with PHE. In the second study, two groups of animals (n = 5) were treated once daily for 8 days with morphine 6 mg kg^{-1}, i.p. Three other groups of animals (n = 5) received chronic dosing of saline i.p. also for 8 days. On day 9, animals treated with chronic morphine received either morphine (3 mg kg^{-1}, i.p., 30 min before formalin) or PHE (100 mg kg^{-1}, p.o., 60 min before formalin, respectively), whereas three saline-treated groups received either a similar administration of saline, morphine (3 mg kg^{-1}, i.p.), or PHE (100 mg kg^{-1}, p.o.).

Data analysis: In all experiments, a sample size of five animals (n = 5) were used. Raw data was calculated as the percentage change in maximum possible effect (% MPE). The time-course curves were subjected to two-way (treatment×time) repeated measures analysis of variance (ANOVA) with Bonferroni's post hoc test. Total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\text{Inhibition (\%)} = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}}\right) \times 100$$

Differences in AUCs were analyzed by ANOVA followed by Student-Newman-Keuls' post hoc test. Doses for 50% of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a + (b - a)}{\left(1 + 10^{(LogED_{50} - X)}\right)}$$

where, X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

The fitted midpoints (ED_{50}s) of the curves were compared statistically using F-test (Miller, 2003; Motulsky and Christopoulos, 2003). GraphPad Prism for Windows version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $p<0.05$ was considered statistically significant.

RESULTS

Phytochemical analysis: The phytochemical analysis of *P. hirsuta* showed it contains alkaloids, flavonoids, tannins and terpenoids with tannins and flavonoids being the most dominant chemical constituents.

Tail-immersion test: All the test drugs caused an increase in the tail withdrawal latency. PHE (30-300 mg kg^{-1}, p.o.,) (Fig. 1a) produced a significant and dose dependent increase in the withdrawal latencies of the tail as depicted in the time-course curve ($F_{3,16} = 5.44$, $p = 0.009$). As shown in Fig. 1b, PHE (300 mg kg^{-1}, p.o.,) increased the withdrawal latency by 43.83±11.62%. Similarly, diclofenac (10-100 mg kg^{-1}, i.p.,) (Fig. 1c) produced a significant anti-nociceptive activity by dose-dependently increasing the tail withdrawal latencies of animals pre-treated with the drug ($F_{3,16} = 10.81$, $p<0.0004$) with the highest dose of 100 mg kg^{-1} causing a percentage increase of 73.75±14.99% as shown in (Fig. 1d). Animals pre-treated with morphine (1-10 mg kg^{-1}, i.p.) also showed a great increase ($F_{3,16} = 15.76$, $p<0.0001$) in their tail withdrawal latencies as observed in the time-course curve in a dose-related manner (Fig. 1e) with the highest dose of 10 mg kg^{-1} given a total anti-nociceptive effect with %MPE of 103.83± 20.46% within the cut-off time of 10 sec (Fig. 1f). Comparison of ED_{50}s obtained by non-linear regression revealed that the extract [ED_{50}:52.12±19.86 mg kg^{-1}] was 26× less potent than morphine [ED_{50}:1.99±0.76 mg kg^{-1}] and 3× less potent than diclofenac [ED_{50}:13.74±5.24 mg kg^{-1}].

Carrageenan-induced mechanical hyperalgesia using randall sellito: On the day of experiment, animals showed baseline withdrawal thresholds of about 60 to 150 g. Two and half hours after carrageenan injection, the ipsilateral paw exhibited marked mechanical hyperalgesia in all experiments which was maintained in vehicle-treated animals throughout the experiment. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect. PHE (30-300 mg kg^{-1}, p.o.) administered 3 h after carrageenan produced a significant and dose-dependent reversal of mechanical hyperalgesia ($F_{3,16} = 25.03$, $p<0.0001$) (Fig. 2a). The highest dose of PHE completely reversed the inflammatory-induced mechanical hyperalgesia with % MPE of 154.79±15.84% as shown in Fig. 2b. The i.p. administration of diclofenac (10-100 mg kg^{-1}) significantly ($F_{3,16} = 17.77$, $p<0.0001$) and

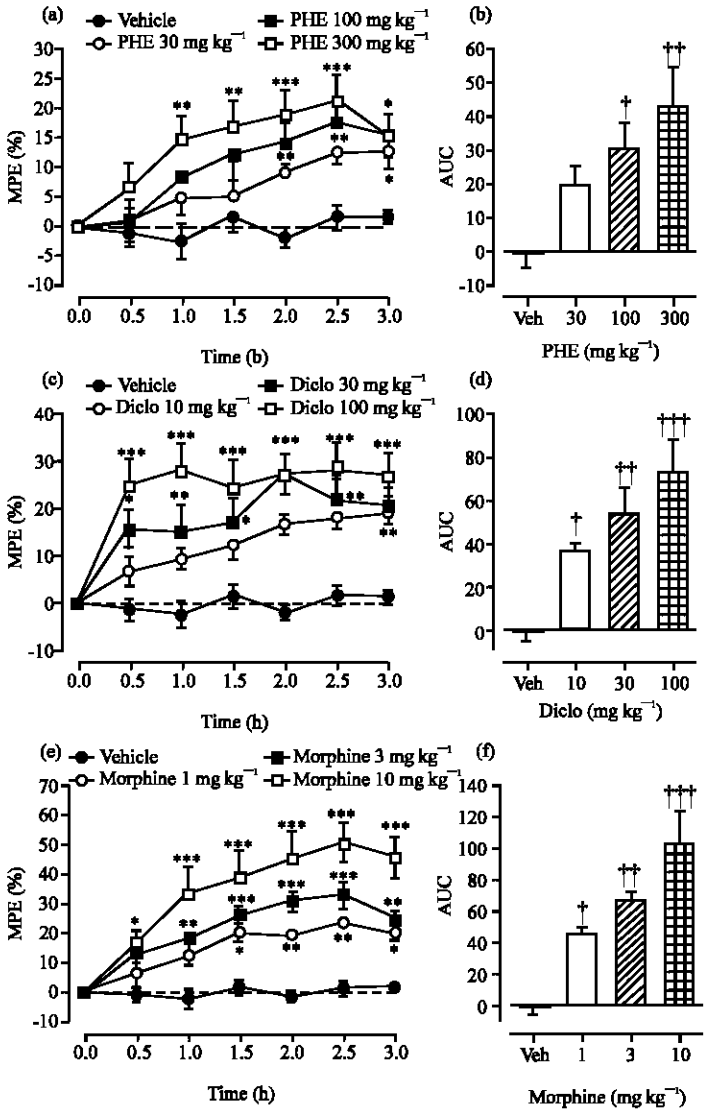

Fig. 1: Effect of PHE (30-300 mg kg^{-1} p.o.), diclofenac (10-100 mg kg^{-1} i.p.) and morphine (1-10 mg kg^{-1} i.p.) on time course curve of tail immersion test (a, c and e) and the AUC (b, d and f). Data was presented as Mean±SEM (n = 5); ***p<0.001; **p<0.01; *p<0.05 compared to vehicle-treated group (two-way ANOVA followed by Bonferroni's post hoc test). †††p<0.0001, †p<0.05 compared to vehicle-treated group (one-way ANOVA followed by Neuman-Keul's post hoc test)

dose dependently relieved the mechanical hyperalgesia as depicted in (Fig. 2c). The highest dose of diclofenac also completely reversed the inflammatory-induced mechanical hyperalgesia with a % MPE of 153.09±16.52% (Fig. 2d). Morphine (1-10 mg kg^{-1}) after i.p. administration similarly antagonised mechanical hyperalgesia significantly ($F_{3,16}$ = 18.31, p<0.0001) in a dose-dependent manner as shown in Fig. 2e; with the highest dose completely reversing the hyperalgesia with % MPE of 178.19±19.83% as shown in the AUC curve (Fig. 2f). When ED_{50}s obtained by non-linear regression were compared, the extract [ED_{50}:141.58±53.95 mg kg^{-1}] was found to be 56× less potent than morphine [ED_{50}:2.55±0.97 mg kg^{-1}] and 9× less potent than diclofenac [ED_{50}:14.69±5.60 mg kg^{-1}].

Acetic acid-induced writhing assay: Table 1 represents the total number of writhes induced by acetic acid, during 20 min of observation, beginning 10 min after the i.p. injection. PHE (30-300 mg kg^{-1}, p.o., 60 min before) significantly ($F_{3,16}$ = 5.87, p = 0.0067) reduced the number of writhing induced by acetic acid in mice. Morphine

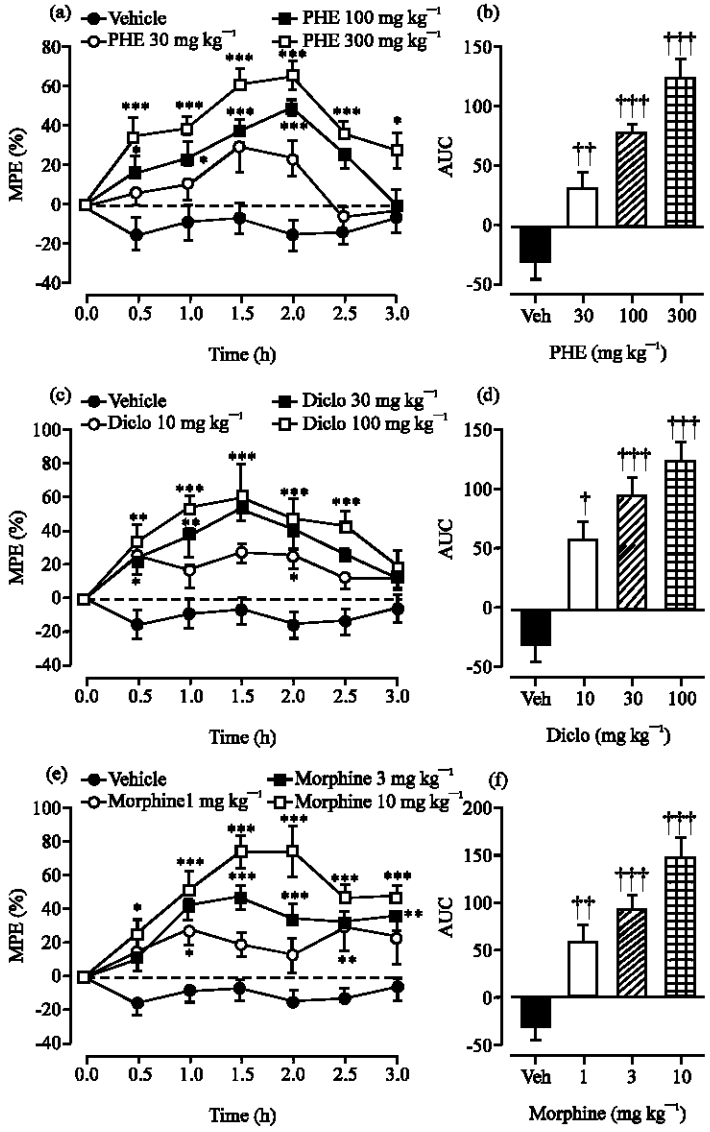

Fig. 2: Effect of PHE (30-300 mg kg^{-1} p.o.), diclofenac (10-100 mg kg^{-1} i.p.) and morphine (1-10 mg kg^{-1} i.p.) on time course curve in carrageenan-induced mechanical hyperalgesia in the rat using the Randall sellito model (a, c and e) and the AUC (b, d and f). Data was presented as Mean±SEM (n = 5); ***p<0.001; ** p<0.01; *p<0.05 compared to vehicle-treated group (two-way ANOVA followed by Bonferroni's post hoc test). †††p<0.0001, †p<0.05 compared to vehicle-treated group (one-way ANOVA followed by Neuman-Keul's post hoc test)

(1-10 mg kg^{-1}, i.p., 30 min before) and diclofenac (10-100 mg kg^{-1}, i.p., 30 min before) both significantly ($F_{3,16}$ = 18.06, p<0.0001) and ($F_{3,16}$ = 13.93, p = 0.0001), respectively, also reduced the number of writhing induced by the acetic acid. Comparison of ED_{50}s obtained by non-linear regression revealed that the extract [ED_{50}:80.20±0.58 mg kg^{-1}] was 127× less potent than morphine [ED_{50}:0.63±0.52 mg kg^{-1}] and 14× less potent than diclofenac [ED_{50}:5.91±0.56 mg kg^{-1}].

Formalin-induced nociception: Formalin administration produced a typical pattern of flinching and licking behavior. The first phase started immediately after administration of formalin and then diminished gradually in about 10 min. The second phase started at about 15 min and lasted until 1 h. Treatment of mice with PHE (10-300 mg kg^{-1}, p.o., 60 min before) (Fig. 3a, b) produced a marked and dose-related inhibition of both phases of formalin-induced nociception first phase

($F_{4,20}$ = 17.35, p<0.0001) second phase ($F_{4,20}$ = 22.729, p<0.0001) with the highest dose causing a maximal inhibition of 83.46±6.67 and 94.56±4.12% of the licking time in the early and late phase, respectively (Fig. 3a, b). Similarly, morphine (1-10 mg kg^{-1}, i.p.) produced marked inhibition of both the neurogenic ($F_{3,16}$ = 18.61, p<0.0001) and inflammatory ($F_{3,16}$ = 18.39, p<0.0001) pain phases (Fig. 3c, d). Morphine, reduced the duration of formalin evoked nociceptive behaviour by a maximum percentage of 90.36±4.68% in the early phase and 96.04±5.50% in the late phase of the formalin test (Fig. 3c, d). Comparison of ED_{50}s obtained by non-linear regression revealed that the extract was 4× more potent in the second phase [ED_{50}:5.36±2.04 mg kg^{-1}] than the first [ED_{50}:21.88±8.34 mg kg^{-1}]. Likewise, morphine was three fold more potent in the second phase [ED_{50}:0.85±0.33 mg kg^{-1}] compared to the first phase [ED_{50}:2.72±1.04 mg kg^{-1}].

Analysis of mechanism of action of PHE: The results presented in Fig. 4a and b show that the pre-treatment of mice with naloxone (2 mg kg^{-1}, i.p., a non-selective opioid receptor antagonist), administered 30 min beforehand, completely and significantly reversed the anti-nociception of both PHE (100 mg kg^{-1}, p.o.) and morphine (3 mg kg^{-1}, i.p.). The adenosine antagonist theophyline (5 mg kg^{-1}) however did not have any significant effect (p<0.05) on the anti-nociceptive effects of both morphine (3 mg kg^{-1}, i.p.) and PHE (100 mg kg^{-1}, p.o.) in the formalin test.

Table 1: Effect of PHE (30-300 mg kg^{-1} p.o), morphine (1-10 mg kg^{-1} i.p.,) and diclofenac (10-100 mg kg^{-1} i.p.,) on acetic acid induced writhing in mice

Drugs	Dose (mg kg^{-1})	No. of writhings
Vehicle	-	39.8±4.02
PHE	30	31.4±8.17
	100	13.6±5.82*
	300	11.6±3.47*
Morphine	1	14.6±5.79***
	3	7.8±2.82***
	10	3.2±1.16***
Diclofenac	10	12.4±5.28***
	30	9.0±2.78***
	100	6.4±4.12***

Values are expressed as Mean±SEM *p≤0.05, **p≤0.01, ***p≤0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls post hoc)

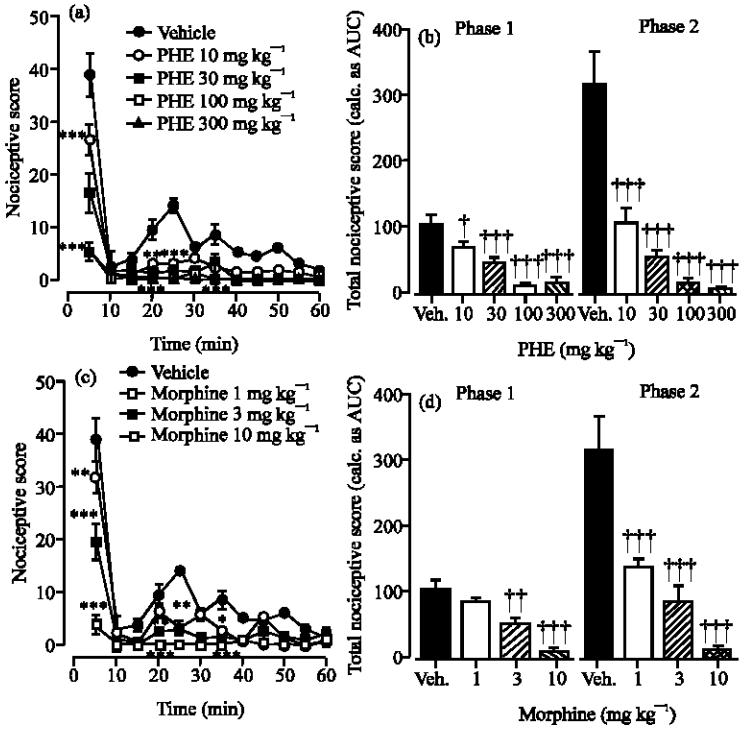

Fig. 3: (a) Effect of PHE (10-300 mg kg^{-1} p.o.) and (c) Morphine (1-10 mg kg^{-1} i.p.) on the time course of formalin induced pain in mice. Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean±SEM (n = 5). *p≤0.05, **p≤0.01, ***p≤0.001 compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni's post hoc); (b and d) the AUC (total response) for phase 1 and phase 2. Each column in b and d represent the mean±SEM, †p≤ 0.05, ††p≤0.01, †††p≤0.001 (one-way ANOVA followed by Newman-Keuls post hoc)

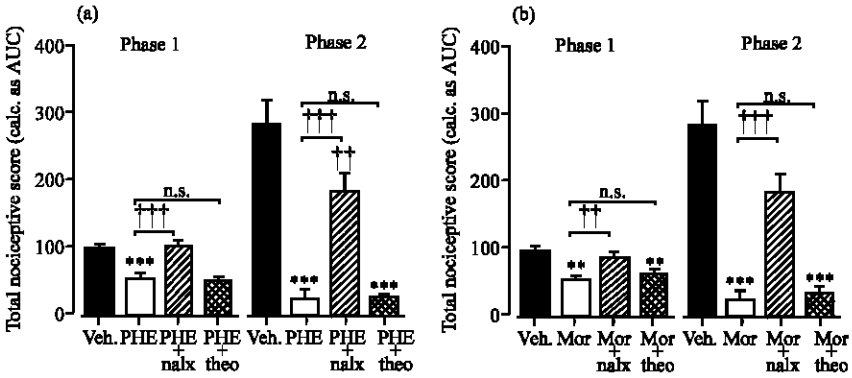

Fig. 4: Effect of intraperitoneal injection of naloxone and theophyline on the anti-nociceptive effect of PHE (10-300 mg kg^{-1} p.o.) and Morphine (1-10 mg kg^{-1} i.p.) on the total nociceptive score of formalin-induced licking test in mice and representing the AUC for phase 1 and phase 2 of formalin-induced pain. Each column represent the Mean±SEM. †p≤0.05, ††p≤ 0.01, †††p≤0.001, *p≤0.05, **p≤0.01, ***p≤0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls post hoc)

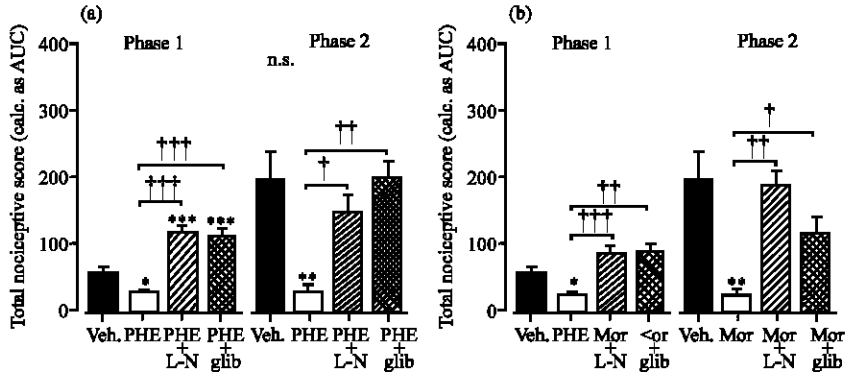

Fig. 5: Effect of intraperitoneal injection of L-NAME and glibenclamide on the anti-nociceptive effect of (a) PHE (10-300 mg kg^{-1} p.o.) and (b) Morphine (1-10 mg kg^{-1} i.p.) on the total nociceptive score of formalin-induced licking test in mice and representing the AUC for phase 1 and phase 2 of formalin-induced pain. Each column represent the Mean±SEM. †p≤0.05, ††p≤0.01, †††p≤0.001, *p≤0.05, **p≤0.01, ***p≤0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls post hoc)

Systemic pre-treatment of mice with L-NAME (a NO synthase inhibitor; 10 mg kg^{-1}, i.p.) significantly and absolutely prevented the anti-nociceptive effect induced by the oral administration of PHE (100 mg kg^{-1}, p.o.) and morphine (3 mg kg^{-1}, i.p.) (Fig. 5a, b) in the formalin test.

Pre-treatment with an ATP-sensitive K^+ channel inhibitor, glibenclamide (8 mg kg^{-1}, p.o.), also prevented the anti-nociception produced by PHE (100 mg kg^{-1}, p.o.) as well as morphine (3 mg kg^{-1}, i.p.) in the formalin test (Fig. 5a, b).

Tolerance studies: Morphine (3 mg kg^{-1}, i.p.) significantly ($F_{5,24}$ = 10.01, p<0.0001 phase 1, $F_{5,24}$ = 10.01, p<0.0001 phase 2) attenuated basal nociceptive response in both phases of formalin test in chronic vehicle-treated animals. However, the same dose of morphine administered at day 9 in animals chronically treated with 6 mg kg^{-1}, i.p. morphine failed to show such effect indicating development of tolerance (Fig. 6). In contrast, 100 mg kg^{-1}, p.o. PHE showed a comparable activity in mice given chronic treatment of either 200 mg kg^{-1}, p.o. PHE or vehicle, indicating lack of tolerance development (Fig. 6). Moreover, 100 mg kg^{-1}, p.o. PHE still demonstrated anti-nociceptive activity in mice chronically treated with morphine, indicating that no cross-tolerance exists with morphine (Fig. 6).

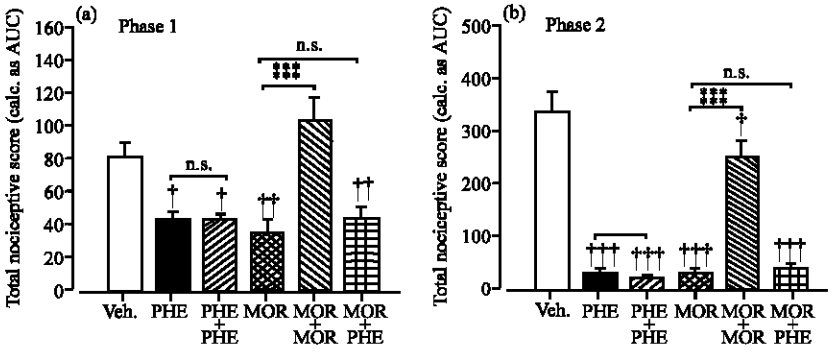

Fig. 6: Effect of PHE (100 mg kg^{-1} p.o.) and morphine (3 mg kg^{-1} i.p.) challenge on mice chronically treated with saline, PHE (200 mg kg^{-1} p.o.) or morphine (6 mg kg^{-1} i.p.) for 9 days on the total nociceptive score of formalin-induced licking test in mice and representing the AUC for phase 1 and phase 2 of formalin-induced pain. Each column represent the mean±SEM. †p≤0.05, ††p≤0.01, †††p≤0.001, *p≤0.05, **p≤0.01, ***p≤0.001 compared to respective controls (one-way ANOVA followed by Newman-Keuls post hoc)

DISCUSSION

This present study has demonstrated that oral administration of an ethanolic extract of the leaves of *Palisota hirsuta* caused potent anti-nociception in the tail immersion, acetic acid-induced writhing, carrageenan-induced mechanical hyperalgesia and the formalin-induced paw licking tests in rodents. This anti-nociceptive effect was reversed by the systemic administration of the non-selective opioid antagonist, naloxone, the NO synthase inhibitor, L-NAME and an ATP-sensitive K^+ channel inhibitor, glibenclamide. The non-selective adenosine receptor antagonist, theophyline however did not alter the anti-nociceptive effect of the extract.

Several behavioural nociceptive tests which differ with respect to stimulus quality, intensity and duration, were employed in evaluating the analgesic effect of PHE in order to obtain holistic picture of the analgesic properties of the extract. The models were selected such that both centrally and peripherally mediated effects were investigated. At the doses tested, the ethanolic extract was shown to have anti-nociceptive activity in all the nociceptive models thus indicating that the extract had both centrally- and peripherally-mediated activities (Vongtau *et al*., 2004).

The writhing response of mice to an intraperitoneal injection of noxious chemical is used to screen for both peripherally and centrally acting analgesic activity. Acetic acid causes pain by releasing endogenous substances and other substances that excite pain nerve endings. The abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins (Bose *et al*., 2007). Diclofenac and other NSAIDs can inhibit the number of writhes in this model by inhibiting cyclooxygenase in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors by blocking the effect or the synthesis and/or release of inflammatory mediators (Panthong *et al*., 2007). It is therefore plausible to suggest that the extract may be acting via mechanisms similar to NSAIDs. However, further experiments may be needed to consolidate this view.

The extract together with morphine and diclofenac also had a significant effect in the tail-immersion test even though this model is known to be more sensitive to centrally acting analgesics (Santos *et al*., 2005). Such analgesic agents elevate pain threshold of animals towards heat and pressure and as such some amount of central activity (spinal and supra spinal mechanisms), (Jain *et al*., 2001) can be conferred on the extract since it exhibited significant activity in this pain model.

The Randall-Selitto paw pressure test which detects the time to movement of an inflamed hind paw to noxious stimuli revealed hyperalgesia in all carrageenan-treated animals. The extract together with morphine and diclofenac at all doses tested, exhibited significant analgesic activity in this pain model which is often used to distinguish between central and peripheral analgesic actions. Inflammation is known to lower the thresholds of various mechanoreceptors and mechanotransduction pathways (Park *et al*., 2008). The stimulus applied in this model of nociception, pressing of a blunt tip into inflamed hind paws, is likely to activate slowly-adapting mechanoreceptors with decreased thresholds, which are predominantly C-fibres located in cutaneous and subcutaneous structures that would have required greater stimulus intensities for activation (Lewin and Moshourab, 2004; Birder and Perl, 1994).

Results obtained in the formalin test showed that both PHE extract and morphine significantly reduced the time spent in licking the injured paw. In this test, the early

phase is considered to be produced by direct activation of nociception neurons by formalin, whereas the late phase reflects pain generated in acutely injured tissue (Tang *et al*., 2007; Hunskaar and Hole, 1987). The licking response induced by formalin, results from a combination of peripheral input and spinal cord sensitization (Tjolsen *et al*., 1992). The intraplantar injection of formalin, releases EAAs, PGE_2, NO and kinins in the spinal cord (Tjolsen *et al*., 1992). Taking this into account, the anti-nociception of *Palisota hirsuta* could be dependent on either peripheral or central sites of action. Centrally acting drugs, such as opioids, inhibit both phases of pain by equally inhibiting the effect produced by prostaglandins released at this level in response to inflammation (Ferreira, 1981; Hunskaar and Hole, 1987; Shibata *et al*., 1989) and by endogenous opioids through their action on the central nervous system. It has been demonstrated (Tjolsen *et al*., 1992) that the late phase in formalin test depends on an inflammatory reaction in peripheral tissue. Peripheral acting drugs such as diclofenac (Rosland *et al*., 1990) which block prostaglandin synthesis reduce nociception mostly in the late phase but can also affect the early stage (Ortiz *et al*., 2008). In fact, the anti-nociceptive effects of PHE as exhibited in the formalin test suggest an involvement at both central and peripheral levels, which further implies that the extract possesses not only anti-nociceptive but also anti-inflammatory activity.

In the present study, the possible mechanism of action of the extract was investigated. The formalin test was selected for this study, since it is more specific and it is possible to identify two distinct phases of nociception (Yin *et al*., 2003; Basile *et al*., 2007) and also since it reflects different pathological processes and it allows the elucidation of the possible mechanism involved in analgesia (Tjolsen *et al*., 1992). The anti-nociceptive effect of PHE was determined in the presence of naloxone, theopylline, L-NAME or glibenclamide. Naloxone, a non selective opioid antagonist, reversed the anti-nociceptive effect of both morphine and the ethanolic leaves extract of PHE in both phases of the formalin test. This finding clearly suggests that activation of opioid receptors and/or an increment of endogenous opioids, either centrally or peripherally, might be involved in the anti-nociceptive effect of PHE (Björkman *et al*., 1990). The anti-nociceptive effect of PHE as well as that of morphine were also blocked by the nitric oxide synthase inhibitor L-NAME, suggesting that the anti-nociceptive action of PHE like morphine involves the activation of the nitric oxide-cyclic GMP pathway at peripheral and/or central levels (Duarte and Ferreira, 1992; Tonussi and Ferreira, 1994; Granados-Soto *et al*., 1995, 1997; Nozaki-Taguchi and Yamamoto, 1998; Islas-Cadena *et al*., 1999). It has been clearly established that NO is a downstream signalling molecule released in response to central analgesics specifically morphine (Cadet *et al*., 2004). Hence, the release of NO or its production is an important step for the anti-nociceptive action of *P. hirsuta* and may contribute for the plant effects against formalin-induced nociception.

Glibenclamide, an ATP-sensitive K^+ channel blocker also blocked the analgesic activity of both PHE and morphine. It is well established that glibenclamide specifically blocks ATP sensitive K^+ channels, with no effect on Ca^{2+} or voltage dependent K^+ channels (Amoroso *et al*., 1990; Edwards and Weston, 1993). Therefore, present data suggest that opening of ATP-sensitive K^+ channels plays a role in the analgesic action of PHE. It is likely that PHE has a mechanism of action similar to diclofenac, metamizol, ketorolac, sodium nitroprusside and morphine all of which activate the nitric oxide-cyclic GMP-K^+ channel pathway (Carrier *et al*., 1997; Rodrigues and Duarte, 2000; Soares *et al*., 2000; Lázaro-Ibáñez *et al*., 2001; Alves *et al*., 2004; Ortiz *et al*., 2002). It is likely that, compounds that open K^+ channels by direct activation like PHE may gain importance as effective pain relievers since these have been shown to be very effective in models of acute and chronic pain (Ocaña *et al*., 2004).

Theophyline, an adenosine receptor antagonist however was not able to modify PHE-induced anti-nociception giving an indication that, the adenosinergic pathway is not involved in the anti-nociceptive effects of PHE.

At this point, it is important to mention that one of the current trends in nociception studies is the search for opioid analgesics acting at opioid receptors outside the central nervous system, with the prospect of avoiding centrally-mediated side effects such as tolerance and dependence (Stein *et al*., 2000). The results reported here suggest that, unlike morphine, PHE does not induce tolerance to its anti-nociceptive effect after chronic administration in the formalin test. Eight days of 6 mg kg^{-1} morphine administration produced significant tolerance in mice treated at day 9 with 3 mg kg^{-1} morphine. In contrast, chronic treatment with 200 mg kg^{-1} PHE did not modify the day 9 anti-nociceptive activity of 100 mg kg^{-1} PHE. The absence of tolerance with PHE treatment cannot be attributed to the use of a low dose, because PHE was chronically administered at the dose maximally active in both phases of the formalin-induced pain. Based on this, it can be speculated that, PHE might have a greater effect on the opioid receptors at the periphery since tolerance is known to be induced centrally. The present study further demonstrates that at doses tested, morphine tolerance does not cross-generalize to PHE.

Overall, the present results demonstrate that the anti-nociceptive effects exhibited by PHE might partially or wholly be due to the stimulation of peripheral opioid receptors through the activation of the nitric oxide-cyclic GMP- ATP-sensitive K^+ (NO/cGMP/K^+ATP)-channel pathway. These findings therefore may contribute to the design of pharmacological strategies directed towards a better management of some painful conditions.

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