ANALGESIC ACTIVITY OF PET ETHER, AQUEOUS, AND HYDRO-ETHANOLIC LEAF EXTRACTS OF *ASPILIA AFRICANA* (PERS) C.D. ADAMS (ASTERACEAE) IN RODENTS

Koffuor George Asumeng^{1*}, Ameyaw Elvis Ofori², Oppong Kyekyeku James³, Amponsah Kingsley Isaac⁴, Sunkwa Andrews¹, Semenyo Samuella Afriyie¹

¹Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

²Department of Biomedical and Forensic Sciences, University of Cape Coast, Cape Coast, Ghana

³Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

⁴Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

*Corresponding Author Email: gkoffuor@yahoo.com

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ABSTRACT

Traditionally, *Aspilia africana* is used in the management of pain in Ghana and most parts of West Africa. This study therefore investigated the analgesic effect of the petroleum ether, aqueous, and hydro-ethanolic leaf extracts of *Aspilia africana* using rodent models. Preliminary phytochemical screening was done on all the extracts, which showed the presence of alkaloids, flavonoids, saponins, tannins, glycosides, phytosterols and terpenoids. The extracts (40-400 mg/kg *p.o.*) were administered to Sprague-Dawley rats and tail flick latencies (Tail flick analgesic model) were measured in a preliminary analgesic study. The order of analgesic efficacy established was hydro-ethanolic > aqueous > petroleum ether extract. Thin layer and high performance liquid chromatographic analyses were carried out on the hydro-ethanolic extract to obtain chromatograms as fingerprints for identification purposes. These revealed seven spots (TLC) and two peaks (HPLC). Acetic acid-induced writhing and Capsaicin-induced nociception analgesic tests were carried out in ICR mice using the hydro-ethanolic leaf extract. This significantly ($P \leq 0.001$) and dose-dependently suppressed the time-course of acetic acid-induced writhing and capsaicin-induced nociception similar to 10 mg/kg Diclofenac sodium ($P \leq 0.001$) and 5 mg/kg, Ketamine ($P \leq 0.001$). In conclusion, the leaf extracts of *Aspilia africana* has significant analgesic activity with the hydro-ethanolic extract being the most potent.

Keywords: Acetic acid-induced writhing, Capsaicin-induced nociception, Acute pain, Tail flick latency.

INTRODUCTION

Pain is a direct response to an untoward event associated with tissue damage, such as injury, inflammation or cancer.¹ However, severe pain can arise independently of any obvious predisposing cause (e.g. trigeminal neuralgia), or persist long after the precipitating injury has healed (e.g. phantom limb pain). It can also occur as a consequence of brain or nerve injury (e.g. following a stroke or herpes infection).² World Health Organization (WHO)³ estimated that approximately 80% of the world population has either no or insufficient access to treatment for moderate to severe pain. The management of pain is currently based on the use of opioids, non-steroidal anti-inflammatory drugs, hypnotics, antidepressants and antiepileptic drugs. Non-NSAID, non-narcotic analgesics such as acetaminophen is also used. These analgesics have numerous side effects ranging from liver failure, typical of the para-aminophenol derivatives like paracetamol; gastric ulcerations which are associated with NSAIDS; and dependence, loss of efficacy in some pain states and tolerance as seen with the narcotic analgesics just to mention a few.⁴ It has become necessary then, to explore alternate ways to provide adequate pain management and one of the ways to achieve this is to screen local herbs which are more readily available for analgesic properties. It has been more than 15 years since the UN recommended the integration of traditional medicine into the orthodox system to back the primary health care programme, thus leading to the now very evident global shift from a health system solely based on orthodox medicine to one where traditional medicine, including the use of herbal medicine is incorporated into the health system. *Aspilia africana* enjoys

several traditional uses. One notable use of this plant is to stop bleeding. It is used in the treatment of rheumatic pain⁵ removal of corneal opacities, induce delivery and in the treatment of anaemia and various stomach complaints.⁶ In Kenya, It is used to kill intestinal worms. In Uganda, it is used to treat gonorrhea.⁷ The methanol extract of the leaves is reported to cure malaria and respiratory problems.⁸ A decoction of the leaves is used to cure eye problem and as a lotion for the face to relieve febrile headache. The medicinal plant contains ascorbic acid, riboflavin, thiamine and niacin. This herb is good sources of minerals such as Ca, P, K, Mg, Na, Fe and Zn.⁹ The aim of the study therefore was to investigate the analgesic activity of the petroleum ether, aqueous and hydro-ethanolic leaf extracts of *Aspilia africana* using rodent models.

MATERIALS AND METHODS

Collection and Identification of Plant

Fresh leaves of *Aspilia africana* were collected from growing *Aspilia africana* plants behind the College of Engineering, Kwame Nkrumah University of Science and Technology (KNUST) in November, 2012 and authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana, where a voucher specimen has been deposited (number KNUST/HM1/2012/S009).

Extraction of Plant Material

The fresh leaves of *Aspilia africana* collected were sun-dried for three days. The dried leaves were powdered using a hammer mill (Polymix Micro Hammer Cutter Mill, Glen

Mills Inc. USA). Three samples of 150 g each of the powdered leaves were extracted by maceration with 1 litre of either Petroleum ether, Distilled water, or 70 % Ethanol. Each extract was concentrated using a rotary evaporator (Model: Rotavapor R-210, Buchi, Switzerland) and dried in a hot air oven (Oven 300 plus series, Gallenkamp, England) at 40°C. The pet-ether extract (PEAA) gave a yield of 1.29 % w/w , the 70 % ethanol extract (EtAA) afforded 9.53% w/w and the aqueous extract (AQAA) gave a yield of 15.95% w/w .

Ethical and Biosafety Considerations

Laboratory study was carried out in a level 2 biosafety laboratory. Protocols for the study were approved by the Departmental Ethics Committee. All activities during the studies conformed to accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All the technical team observed all institutional biosafety guidelines for protection of personnel and laboratory.

Experimental Animals

Male ICR mice (15–25 g) and female Sprague-Dawley rats (50–65 g) were obtained from Noguchi Memorial Research Institute, Legon, Accra, Ghana and kept in the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology Kumasi, Ghana. They were housed in groups of 5 or 6 in stainless steel cages with soft wood shavings as their bedding. The animals were kept under ambient dark/light cycle and humidity. Water and a normal commercial pellet diet (GAFCO, Tema, Ghana) were provided for the animals *ad libitum*.

Identifying *Aspilia africana* Extract of Highest Analgesic Activity

The tail immersion test by Thirumal et al.,¹⁰ with modifications was used. Female Sprague-Dawley rats (50–65 g) were grouped into five, (n=6) and allowed to adapt to the cages for 30 minutes before experimentation. Baseline pain thresholds were taken by immersing the tail of each animal in a water bath maintained at 55°C. The time of tail flick (reaction time) was recorded (cutoff point of 10 s). The average reaction times were calculated. The groups were treated with either PEAA (40, 100, or 400 mg/kg, *p.o.*), AQAA (40, 100, or 400 mg/kg, *p.o.*), EtAA (40, 100, or 400 mg/kg, *p.o.*), Diclofenac (10 mg/kg, *i.p.*) or 10 ml/kg Distilled water (control group). The reaction times were again recorded for each animal 30 minutes after *i.p.* treatments or 1 hour after oral treatments. The percentage maximum possible effect (MPE) was calculated for each animal using the formula:

$$\%MPE = \frac{T_2 - T_1}{T_0 - T_1} \times 100$$

Where T1 and T2 are pre and post drug reaction times and T0 is the cut-off time

Establishing Analgesic Activity of EtAA

Acetic Acid-Induced Writhing Test

The writhing test by Woode et al.¹¹ was further used to evaluate the analgesic activity of the EtAA. Five groups of male mice, (n=6), were treated with either EtAA (40, 100, or 400 mg/kg, *p.o.*), Diclofenac (10 mg/kg, *i.p.*) or 10 ml/kg Distilled water. These treatments were made 30 minutes (for *i.p.* treatment) or 1 hour (for *p.o.* treatment) prior to acetic acid (1 ml, 0.6%; *i.p.*) administration to induce writhing. Each mouse was placed individually in a testing chamber. Writhing (stretching of the abdomen with simultaneous stretching of at

least one hind limb) was captured for 30 minutes by a camcorder which was placed directly opposite a mirror inclined at 45° below the testing chambers. Tracking of the behavior was done using the public domain software JWatcher™ Version 1.0 (University of California, LA, USA and Macquarie University, Sidney, Australia, available at <http://www.jwatcher.ucla.edu/>). The number of writhes was recorded for each animal. A dose–response curve was also plotted to determine the significant anti-nociceptive dose.

Capsaicin- Induced Nociceptive Model

The capsaicin-induced nociceptive test was performed as described earlier¹² with some modifications. Five groups of male mice (n=6) were treated with either EtAA (40, 100, or 400 mg/kg, *p.o.*), Ketamine (5 mg/kg, *i.p.*) or 10 ml/kg Distilled water. Thirty minutes (for *i.p.* treatment) or 1 hour (for *p.o.* treatment) thereafter, mice were injected intraplantarly with capsaicin (20 µl dissolved in 0.5 % ethanol; 1.6 µg/paw) to induce nociception. The nociceptive behaviour (biting/licking of the injected paw) following capsaicin injection was captured for 15 minutes by a camcorder (Everio™, model GZ-MG1300, JVC, Tokyo, Japan) placed directly opposite a mirror inclined at 45° below the testing chambers. The behavior of the mice was tracked using the public domain software JWatcher™, Version 1.0 (University of California, LA, USA and Macquarie University, Sidney, Australia, available at <http://www.jwatcher.ucla.edu/>) to obtain the frequency and duration of biting/licking per 5 minutes. A nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking.

Phytochemical Analysis

PEAA, AQAA and EtAA were subjected to phytochemical screening using standard techniques of phytochemical analysis as described by Sofowora (1993),¹³ Harbone (1998),¹⁴ and Trease and Evans (1989).¹⁵

Thin Layer Chromatography

Aluminium precoated silica gel plates 60 F₂₅₄ (0.25 mm thick) was cut to an appropriate size so as to fit in a chromatank. EtAA (5 mg) to be analysed by TLC was constituted in ethanol (95 %) and applied on the TLC plates as spots with the aid of capillary tubes at one end of the plate in a straight line about 2 cm above the edge and 1.5 cm away from the margins. The spots were dried and the plates placed inside a chromatank saturated with the mobile phase chloroform/methanol (98:2). The one way ascending technique was used to develop the plate. The zones on TLC plates corresponding to separated compounds were detected under UV light 254 nm and 365 nm and also by spraying with anisaldehyde 0.5 % w/v in HOAc/H₂SO₄/ MeOH (10:5:85) followed by heating at 105°C for 5–10 minutes.

High Performance Liquid Chromatography (Qualitative Determination)

Approximately 2 ml of a 0.1 % w/v ethanolic (absolute) solution of EtAA was transferred into a 1 cm thick cuvette and placed in a double beam UV spectrophotometer (T90 + UV/Visible Spectrophotometer, PG Instruments Ltd., UK) to obtain a UV/Visible spectrum. The wavelength of maximum absorption was selected from the spectrum and used as the wavelength for the HPLC determination. The HPLC chromatograph consisted of a pump (LC-10AD, Shimadzu Corporation, Kyoto, Japan) connected to a UV detector

(PerkinElmer 785A UV/Visible detector, USA), using a phenyl column (Zorbax, 3.0 x 150 mm x 3.5 microns) and methanol: water (90:10) as the stationary phase and mobile phase respectively. A 20 μ L quantity of the sample was analyzed isocratically at a wavelength of 278 nm (flow rate of 1 ml/min) and a chromatogram obtained.

Data Analysis

Time-course curves were obtained for the tail flick test by using Graph Pad Prism 5 for Windows (Graph Pad Software, San Diego, CA, USA). AUC values obtained from the tail flick test curves, the acetic acid-induced writhing test and the capsaicin-induced nociceptive test were subjected to one-way analysis of variance with Dunnet's multiple comparisons *post hoc* test for statistical significance. P value ≤ 0.05 was considered statistically significant in all analysis.

Table 1: Phytochemical Constituents of extracts of the leaves of *Aspilia africana*

Test	PEAA	AQAA	EtAA
Flavonoids	+	+	+
Phytosterols	+	-	+
Alkaloids	+	+	+
General test for glycosides	+	+	+
Saponin glycosides	-	+	+
Tannins	-	+	+
Anthracene glycosides	-	-	-
Cyanogenetic glycosides	-	-	-
Terpenoids	+	-	+

+: present, -: absent, Pet-ether extract (PEAA), Aqueous extract (AQAA), Ethylacetate extract (EtAA)

Table 2: R_f values obtained for the hydro-ethanolic extract of the leaves of *Aspilia africana* (EtAA) in a TLC analysis

Spots	R_f
1	0.095
2	0.214
3	0.595
4	0.762
5	0.857
6	0.929
7	0.976

Solvent system EtOAc: CHCl_3 : Glacial CH_3COOH (3:1:1), visualizing under UV 254 nm

Figure 1: *Aspilia africana* growing in the wild

Figure 2: HPLC chromatogram of EtAA. Stationary phase: SB phenyl column; mobile phase: CH_3OH : H_2O (90:10); $\lambda = 278\text{nm}$; Flow rate: 1 ml/min; Temperature: ambient

	PEAA	AQAA	EtAA	Diclofenac	Distilled water
AUC	52.33 $\pm$ 13.5 ***	60.42 $\pm$ 17.7 ***	73.60 $\pm$ 19.6 ***	149.30 $\pm$ 39.6 ***	5.79 $\pm$ 2.84

Figure 3: Effect of PEAA, AQAA, EtAA (40 mg/kg, *p.o.*) and Diclofenac (10 mg/kg, *i.p.*) on the time course curves of tail flick latencies and the total nociceptive score (calculated as AUC) in the mice. Data are expressed as mean $\pm$ SEM, (n=5). * $P < 0.001$ compared to vehicle-treated group (One-Way Analysis of Variance (ANOVA) followed by Dunnet's *post hoc* test)**

Figure 4: Effect of PEEA, AQAA, EtAA (100 mg/kg, *p.o.*) and Diclofenac (10 mg/kg, *i.p.*) on the time course curves of tail flick latencies and the total nociceptive score (calculated as AUC) in the mice. Data are expressed as mean $\pm$ SEM. (n=5). *** $P \leq 0.001$ **** $P \leq 0.0001$ compared to vehicle-treated group (One-Way Analysis of Variance (ANOVA) followed by Dunnet's *post hoc* test)

Figure 5: Effect of PEEA, AQAA, EtAA (400 mg/kg, *p.o.*) and Diclofenac (10 mg/kg, *i.p.*) on the time course curves of tail flick latencies and the total nociceptive score (calculated as AUC) in the mice. Data are expressed as mean $\pm$ SEM. (n=5). *** $P \leq 0.001$ compared to vehicle-treated group (One-Way Analysis of Variance (ANOVA) followed by Dunnet's *post hoc* test)

Figure 6: Effect of EtAA (40-400mg) and diclofenac (10mg/kg) on acetic acid-induced abdominal writhes using One-Way Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparison Test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ compared to vehicle-treated group

Figure 7: Effect of an EtAA (40 - 400 mg/kg), Diclofenac (10 mg/kg) and Ketamine (5 mg/kg) on capsaicin-induced nociception using One-Way Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparison Test. ** $P \leq 0.01$; *** $P \leq 0.001$ compared to vehicle-treated group

RESULTS

Tail Immersion Test

PEAA, AQAA and EtAA significantly produced an increase in the tail flick latency [$P \leq 0.001$: (Figures 3, 4 and 5)]. PEAA, AQAA and EtAA (40-400 mg/kg) increased the tail flick latencies dose-dependently with a maximal effect at the dose of 400 mg/kg for all the extract treated groups. Diclofenac (10 mg/kg) also produced significant anti-nociception ($P \leq 0.001$: Figures 3, 4 and 5). EtAA (40-400 mg/kg) exhibited the highest anti-nociceptive effect thus recording the highest percentage maximum possible effect for all doses used.

Acetic acid-induced Writhing Test

The acetic acid injected intraperitoneally produced writhing, seen as an extension of the abdomen with simultaneous stretching of at least one of the hind limbs. Control mice pre-treated with distilled water exhibited the highest number of writhes. EtAA and Diclofenac significantly suppressed ($P \leq 0.05$) the time-course of acetic acid-induced writhes (Figure 6).

Capsaicin- Induced Nociceptive Model

Capsaicin induced nociceptive response that was manifested by the mice as biting and licking of the injected paw. EtAA (40-400 mg/kg) produced dose-dependent anti-nociception of capsaicin-induced neurogenic pain ($P \leq 0.001$) similar to that produced by 5 mg/kg Ketamine ($P \leq 0.001$) (Figure 7).

Phytochemical Analysis

The phytochemical screening showed the presence of alkaloids, flavonoids and glycosides in all three extracts (Table 1). Phytosterols and terpenoids were present in the pet-ether and ethylacetate extracts whereas tannins and saponins were present only in the aqueous and ethylacetate extracts. Anthracene and cyanogenetic glycosides were absent in all three extracts.

Thin Layer Chromatography (TLC)

The TLC chromatogram of EtAA showed seven (7) separated components with R_f values presented in Table 2.

High Performance Liquid Chromatography (HPLC)

The chromatogram obtained from the HPLC test on EtAA (Figure 2) consists of two prominent peaks suggesting the presence of chromophoric elements in the extract.

DISCUSSION

Aspilia africana has been used in the local communities of Ghana for analgesia. In the tail flick test the three leaf extracts (PEAA, AQAA, EtAA) of the plant produced anti-nociception against thermal pain. Although all the extracts showed significant dose-dependent analgesic activity at all dose levels, EtAA was identified as the most efficacious. The tail immersion test is a sensitive and useful test for demonstrating the analgesic activity of compounds. The mechanism of pain in this model occurs at the level of the spinal cord and supraspinal structures^{16,17}. Therefore, it can be suggested that the analgesic activity of the extracts, PEAA, AQAA and EtAA and Diclofenac in this model was centrally mediated. In order to further evaluate the anti-nociceptive properties of EtAA, acetic acid-induced writhing test, another behavioral animal model of nociception of different stimulus, intensity and duration was employed. This test induces peritoneal inflammation in mice. It is a sensitive method for screening the anti-nociceptive effect of compounds but with some limitations however, in that it may not be very specific since writhing can be suppressed by other non-analgesic drugs such as muscle relaxants and psychoactive drugs.^{18,19} Nociception induced in this model is easily inhibited by non-steroidal anti-inflammatory drugs as well as centrally acting analgesics such as opioids.²⁰ Acetic acid is known to indirectly cause a release of endogenous nociception mediators such as bradykinin, substance P, serotonin, histamine, sympathomimetic amines, prostaglandins (PGE2 and PGF2 α) and pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and IL-8.²⁰ EtAA produced a significant reduction in abdominal writhing in the mice. Its mechanism of analgesia can be attributed to its anti-inflammatory activity which involves the inhibition of mediators such as bradykinin, prostaglandins and substance P or the inhibition of the receptors of the mediators. Diclofenac used as a standard drug in the assay showed significant analgesic effect via its anti-inflammatory mechanisms which involve blocking the cyclooxygenase pathway.² A mechanistic-analgesic model, the capsaicin test was used to determine possible mechanisms of action of EtAA. EtAA produced anti-nociception in this model. Capsaicin, a 8-methyl-N-vanillyl-6-nonenamide compound belonging to the vanilloid family produces nociception by binding to capsaicin (vanilloid or TRPV1) receptors, a ligand-gated non-selective cation channel present on primary sensory neurons.^{21,22} This vanilloid receptor is also activated by heat above the threshold of 45°C and pH below 5.5.² The model is suitable for pain arising from noxious activities C-fibres and A δ afferent neurons as well as polymodal nociceptors and warm

thermoceptors.²³ This throws light on the possible mechanism of analgesia of EtAA. EtAA possibly produces antinociception via the TRPV1 nociceptive pathway by inhibiting the receptors or the neuropeptides, excitatory amino acids and nitric oxide that are produced after the TRPV1 activation. This mechanism may partially be responsible for the observed anti-nociception of EtAA in the tail flick and acetic acid-induced writhing tests as these tests alter cellular temperature and pH: events that can activate the TRPV1 receptors.^{24, 25} Ketamine is a non-competitive antagonist at glutamate receptors (N-methyl-D-Aspartate). It also blocks voltage-sensitive calcium channels and depresses sodium channels which mediate release of peptides such as substance P which can activate the vanilloid receptor.² Its wide range of anti-nociceptive activity contributes to its significant effect observed in this model. The presence of flavonoids, alkaloids, phytosterols, terpenoids, tannins, glycosides and saponins in PEAA, AQAA and EtAA confirmed previous phytochemical tests done on the plant.²⁶ The extract with the highest nociceptive activity (EtAA) was found to contain flavonoids, phytosterols, alkaloids, saponins, tannins and triterpenoids (Table 1). The analgesic and anti-inflammatory effects of flavonoids, steroids, alkaloids and tannins have been reported²⁷; hence the analgesic effect produced by the extract (EtAA) may be attributed individually or collectively to these metabolites. A large number of naturally occurring alkaloids with antinociceptive activity have been reported. Some aporphinoid alkaloids found to exhibit antinociceptive properties includes pronuciferine, glaucine, nuciferine and pukateine.²⁸ A number of plant derived flavonoids are also reported to produce significant antinociception in acetic acid-, formalin and capsaicin-induced nociceptive response.²⁸ Since much work has not been done on the plant, this plate developed can be a very good standard in the identification of subsequent species of the plant. The HPLC (High Performance Liquid Chromatography) analysis performed, using a UV detector at wavelength 278 nm showed two resolved peaks observed on the chromatogram. These peaks suggest that secondary metabolites with chromophoric groups are present in the extract. This chromatogram was developed at ambient temperature using a mobile phase of methanol: water (90:10) and a flow rate of 1 ml/min on a reverse column stationary phase. The chromatogram obtained can be employed as a qualitative tool for the quality control of the hydro-ethanolic leaf extract of *Aspilia africana* (Pers) C.D. Adams (Asteraceae). The extracts exerted anti-nociceptive properties in the various models employed and this may be due to the presence of secondary metabolites acting individually or in concert. These tests i.e. phytochemical screening, TLC and HPLC standardize the extracts. The extracts exerted antinociceptive properties in the various models employed and this may be due to the presence of secondary metabolites acting individually or in concert.

CONCLUSION

The petroleum ether, aqueous, and hydro-ethanolic leaf extracts of *Aspilia africana* have analgesic activity with the hydro-ethanolic extract having the highest activity. These can therefore be used in the management of acute pain.

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Authors' Contributions

George Asumeng Koffuor and Elvis Ofori Ameyaw, wrote the protocol, supervised the study, performed the statistical analysis, and wrote the first draft of the manuscript. James Oppong Kyekyeku supervised/managed the TLC and HPLC analyses of the study. Andrews Sunkwa and Samuella Afriyie Semenyo managed data collection, the literature searches and laboratory work. All authors read and approved the final manuscript.

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