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

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INTRODUCTION

Pain is a direct response to an untoward event associated with tissue damage, such as injury, inflammation or cancer. 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).

World Health Organization (WHO) 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 acetylsalicylic acid, ibuprofen, paracetamol; and代码s, cer, are 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. 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.

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...
Establishing Analgesic Activity of EtAA

Acetic Acid-Induced Writhing Test

The writhing test by Woode et al.\textsuperscript{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 JWWatcher™ 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 earlier\textsuperscript{12} 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 JWWatcher™, 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),\textsuperscript{13} Harborne (1998),\textsuperscript{14} and Trease and Evans (1989).\textsuperscript{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 spots on TLC plates corresponding to separated compounds were detected under UV light 254 nm (T90 + W10AD, Shimadzu) 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 μL 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

<table>
<thead>
<tr>
<th>Test</th>
<th>PEAA</th>
<th>AQAA</th>
<th>EtAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>General test for glycosides</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saponin glycosides</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthracene glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyanogenetic glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Spots</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.095</td>
</tr>
<tr>
<td>2</td>
<td>0.214</td>
</tr>
<tr>
<td>3</td>
<td>0.595</td>
</tr>
<tr>
<td>4</td>
<td>0.762</td>
</tr>
<tr>
<td>5</td>
<td>0.857</td>
</tr>
<tr>
<td>6</td>
<td>0.929</td>
</tr>
<tr>
<td>7</td>
<td>0.976</td>
</tr>
</tbody>
</table>

Solvent system EtoAc: Glacial CH3COOH (3:1:1), visualizing under UV 254 nm

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**Figure 1:** Aspilia africana growing in the wild

**Figure 2:** HPLC chromatogram of EtAA. Stationary phase: SB phenyl column; mobile phase: CH₃OH: H₂O (90:10); λ= 278nm; Flow rate: 1 ml/min; Temperature: ambient

**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 ± 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 PEAA, 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 ± SEM, (n=5). ***P ≤ 0.001 ****P ≤ 0.0001 compared to vehicle-treated group (One-Way Analysis of Variance (ANOVA) followed by Dunnet’s post hoc test)

Figure 5: Effect of PEAA, 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 ± 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 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 ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 compared to vehicle-treated group
High Performance Liquid Chromatography (HPLC)
The phytochemical screening showed the presence of components with $R_f$ values presented in Table 2.

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.
thermoceptors. 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 antinociception 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. 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. Its wide range of anti-nociceptive activity contributes to its significant effect observed in this model. The presence of flavonoids, alkaloids, phytoesters, terpenoids, tannins, glycosides and saponins in PEA, AQA and EtAA confirmed previous phytochemical tests done on the plant.

The extract with the highest nociceptive activity (EtAA) was found to contain flavonoids, phytoesters, alkaloids, saponins, tannins and triterpenoids (Table 1). The analgesic and anti-inflammatory effects of flavonoids, steroids, alkaloids and tannins have been reported, 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 pronunciiferine, glaucine, nuciferine and pukateine. A number of plant derived flavonoids are also reported to produce significant antinociception in acetic acid-, formalin and capsaicin-induced nociceptive response. 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 anti-nociceptive 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 efficacy. These can therefore be used in the management of acute pain.

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Authors’ Contributions

George Asumeng Koffuor and Elvis Ofri 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 Samuela Afrifye Semyeno managed data collection, the literature searches and laboratory work. All authors read and approved the final manuscript.

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