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Antinociceptive effects of an ethanolic extract of *Desmodium adscendens*: possible involvement of opioidergic, adrenergic, potassium channels and serotoninergic pathways

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Abstract

Background: Pain is a major symptom usually associated with most disease states. Despite the existence of many therapies, the management of pain remains unsatisfactory globally. Medicinal plants have been used since medieval times and are still being used today for treating some ailments. *Desmodium adscendens* is used traditionally for the treatment of epilepsy, pain, and inflammatory conditions. However, data on its effect on pain is very scanty.

Objective: This study sought to investigate the antinociceptive effect of an ethanolic extract of *D. adscendens* in rodents.

Methods: The pulverized whole plant material of *D. adscendens* was extracted by cold maceration with 70% ethanol. Chemical, thermal, and neuropathic pain were induced in rodents. The possible mechanisms of analgesia of the extract were also investigated.

Results: The extract of *D. adscendens* (DAE) attenuated acetic acid-induced writhing ($p = 0.0012$), ameliorated formalin-induced nociceptive pain in both the first ($p = 0.0058$) and second phases ($p = 0.0116$), increased the percent maximal possible effect (% MPE) in the hot plate test ($p < 0.0001$) and significantly reduced paclitaxel-induced neuropathic pain in both thermal hyperalgesia ($p < 0.0001$) and cold allodynia ($p = 0.0024$). The analgesic effect exhibited by DAE was significantly reversed in the presence of naloxone, glibenclamide, ondansetron, prazosin, and yohimbine. However, the analgesic effect of DAE was not significantly affected by theophylline, atropine, L-Nitro-arginine methyl ester (L-NAME), and nifedipine.

Conclusion: The ethanolic extract of *D. adscendens* inhibited chemical, thermal, and paclitaxel-induced neuropathic nociception. The DAE may be acting through the opioidergic, adrenergic systems, adenosine triphosphate (ATP)-sensitive K^+ channels, and the serotoninergic pathways to ameliorate pain in murine models.

Keywords: *Desmodium adscendens, analgesia, paclitaxel, hyperalgesia, allodynia*

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INTRODUCTION

Pain is the major symptom usually associated with most disease states which alert a patient to seek medical attention [1]. The International Association for the Study of Pain (IASP), defines pain as "an unpleasant sensory or emotional experience associated with actual or potential tissue damage or described in terms of such damage"[2]. Pain management remains unsatisfactory despite countless efforts and therapies to resolve it [3]. This has always been a global burden and its prevalence, especially among Africans, in recent findings is on the rise [4,5]. Despite the efficacy of current conventional analgesics in use, they are usually associated with deleterious side effects with tolerance built over time [6]. When pain is not managed properly, the associated stress can reduce the quality of life, affect mental health and complicate other disease conditions. In this regard,

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alternative options of pain management are being investigated and the use of traditional medicinal plants as potential efficacious analgesics with minimal adverse effects are being considered [7,8]. Medicinal plants have been used since medieval times for treating some ailments and are still being used today all over the world [9]. *Desmodium adscendens* (Sw) DC var adscendens (*D. adscendens*) is an example of such plants and is extensively used traditionally in Brazil, Peru and the coast of West Africa. It is commonly known as 'Sweetheart' and in Ghana, it is known by the Akans as 'Akwamfanu' or 'Nkatenkate' [10-12]. In folkloric medicine, the leaves of *D. adscendens* are used to treat a range of conditions including asthma, pain, central nervous system disorders, venereal diseases, diarrhoea, excessive urination and ovarian inflammations [13]. Previous studies on various parts of the plant have revealed anti-asthmatic properties [14], antipsychotic, anticonvulsant effects [15,16] as well as anti-oxidant [17] and immunostimulatory effects [18]. Although the analgesic effect of *D. adscendens* using acetic-acid induced writhing has been reported, it was not conclusive enough to scientifically justify and establish it as an analgesic [19]. Additionally, the possible mechanism of this analgesic effect has also not been established. Therefore, based on the folkloric use of the plant in the management of pain and previous report on anti-nociceptive effects in the acetic acid-induced writhing test, this study sought to further investigate the anti-nociceptive effect of the ethanolic extract of *D. adscendens* in peripheral and centrally-mediated pain in selected animal models. This study tested if the extract of *D. adscendens* (DAE) had effect on neuropathic pain and evaluated the possible mechanisms of DAE's analgesic action.

MATERIALS AND METHODS

Study design

We conducted an experimental laboratory-based study using rodent subjects to ascertain the functional effects of *D. adscendens* on pain.

Drugs and chemicals

The chemicals and reagents used in this study included acetic acid (British Drug House, England); formalin (British Drug House, England); theophylline anhydrous (API Ernest Chemists, Ghana); diclofenac (Troge Medical GMBH, Germany); morphine sulphate (Stroup, Belgium); naloxone hydrochloride (Hamelin Pharma Plus, Germany); yohimbine (API Ernest Chemists, Ghana); pentylenetetrazole (API Ernest Chemists, Ghana); glibenclamide anhydrous (API Ernest Chemists, Ghana); L-Nitro-arginine methyl ester (L-NAME); ondansetron (Bayer Pharmaceutical, Germany); nifedipine (Adalat®LA) (Bayer Pharmaceutical, Germany); prazosin (Hypovase™) (Pfizer Pharmaceutical, Germany); atropine sulphate (Hamelin Pharma Plus, Germany); paclitaxel (Intaxel®) (Fresinius Kabi, India); and pregabalin (Lyrica®) (Pharmaceutical, Germany).

Animals

Male imprint control region (ICR) mice (20 - 30 g) and male Sprague-Dawley rats (150 - 200 g) were obtained from Noguchi Memorial Institute for Medical Research and maintained at Animal House, Centre for Plant Medicine Research, Mampong-Akuapem, Ghana. The animals were housed in groups of five in stainless steel cages (34 x 47 x 18 cm) with softwood shavings as bedding, fed with normal commercial pellet diet (AgriCare, Kumasi, Ghana) and allowed water *ad libitum.* They were maintained in a facility with temperature 25 ± 2 °C, relative humidity 60 - 70%, and with 12-h light-dark cycle. All animal procedures and techniques used in these studies were according to guidelines from the Animal Care and Use Committee (IACUC), College of Health Sciences, the University of Ghana with protocol number NIACUC-2017-06-2R. Animals were euthanized using liquid chloroform. Four to six mice (same group) were placed in a cotton padded euthanasia chamber with enough floor space and 6 mL chloroform was delivered into the chamber and closed for 2 min. Death was verified by the assurance of cessation of respiratory and cardiovascular movements.

Plant collection and extraction

Samples of the *D. adscendens* whole plant were collected from the Aburi Botanical Gardens, Ghana, in July 2015. Approval for the collection was provided by the curator of the garden. The plant was identified by a botanist during the collection and authenticated at the Ghana Herbarium, Department of Plant and Environmental Biology, University of Ghana, where a voucher specimen (PA02/UGSOP/GH15) was lodged. The samples of the collected plant were air-dried for 7 days and pulverized. Five kilograms of the powder were macerated at room temperature with 10 L of 70% v/v of ethanol in water. The extract was filtered off every other day and replaced with fresh solvent for 14 days. The various hydro-ethanolic extracts were combined and concentrated on a rotary evaporator (Buchi Rotavapor® R-300, Switzerland) under reduced pressure to remove ethanol. The resulting extract was then placed on a water bath to remove all traces of water to obtain DAE. A 10% w/w yield was obtained and the dried sample of DAE was kept in a desiccator at room temperature until use.

Acetic acid-induced writhing

The test was carried out as previously described [20,21]. Male ICR mice were randomly divided into 7 groups and kept in the experimental environment to acclimatize for 7 days. Each group received either the distilled water (10 mL/kg, *p.o.*), DAE [100, 300 and 1000 mg/kg, per os (*p.o.*)] or diclofenac [10, 30 and 100 mg/kg, intraperitoneal (*i.p.*)] 60 min prior to intraperitoneal administration of acetic acid (0.6%, 10 mL/kg). The mice were then placed individually in a testing chamber (a Perspex chamber, 15 × 15 × 15 cm). The writhing behavioural responses were captured for 30 min for analysis by a camera. Tracking of the behaviour was done using Behavior Tracker® to obtain the frequency of writhes per 5 min, starting immediately after acetic acid

administration. A nociceptive score was determined for each 5 min time block. These data were represented in a time course and the area under the curve (AUC) plotted and analyzed.

Formalin-induced licking

The formalin test was carried out as previously described [22]. Male ICR mice were randomly divided into 7 groups and kept in the experimental environment to acclimatize for 7 days. Each group received either the distilled water (10 mL/kg, *p.o.*), DAE (100, 300 and 1000 mg/kg, *p.o.*), morphine (1, 3 and 10 mg/kg, *i.p.*) or diclofenac (10, 30 and 100 mg/kg, *i.p.*) 60 min before intraplantar injection of 10 µL of 5% formalin into the right hind paw. The animals were immediately placed into the testing chamber, and their nociceptive behaviours scored. The pain response was scored for 1 h, starting immediately after formalin injection. A nociceptive score was determined for each 5 min time block by measuring the amount of time spent biting/licking of the injected paw. Tracking of the behaviour was done using public domain software Behavior Tracker®. Data were expressed and analysed as the mean ± standard error (SEM) of scores between 0 - 10 min (first phase) and 10 - 60 min (second phase) after formalin injection.

Hot plate test

This method first described by Eddy et al. in 1953 was used with no modifications. Male ICR mice (n = 5) were placed on a hot plate (Model 7280, Ugo Basile Inc., Italy) heated to 52 ± 0.5 °C and the baseline reaction time of the animals to nociceptive responses (licking/shaking of the paws, jumping) recorded as baseline reaction latency. They were treated with the test preparations (similar to that described for the writhing test and formalin test above) and the reaction times taken again at 0.5-, 1- and 2-h intervals after a latency period of 30 min following the administration of the control group vehicle (10 mL/kg, *p.o.*), DAE (100, 300 and 1000 mg/kg, *p.o.*), morphine (1, 3 and 10 mg/kg, *i.p.*). A cut-off reaction time was set at 20 sec to prevent damage to tissues of the foot. The per cent maximal possible effect (%MPE) was calculated by the following formula:

$$\%MPE = \left[\frac{Posttreatment\ Latency - Pretreatment\ Latency}{Cut\ off\ time - Pretreatment\ latency}\right] \times 100$$

Mechanism of action DAE using the Hotplate test. In these experiments, mice were pre-treated with receptor-specific antagonists at doses selected and timing of drug administration were based on data from literature before administration of test drug (DAE or standard reference drug) [6]. Treated mice were then subjected to the hot plate test to determine some possible mechanisms by which the extract might exert its analgesic effect.

Possible involvement of the opioid system. The standard reference drug used for this test was morphine. The respective median doses of DAE (300 mg/kg, *p.o.*) and morphine (3 mg/kg, *i.p.*) were selected for this study. Male ICR mice were pre-treated with naloxone (a non-selective opioid receptor antagonist at 2 mg/kg, *i.p.*) and after 15 min received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle (10 mL/kg, *p.o.*). The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after the administration of morphine

Possible involvement of the nitric oxide pathway. Male ICR mice were pretreated with L-NAME (10 mg/kg, *i.p.*) and after 15 min received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Possible involvement of adenosine triphosphate (ATP)-sensitive K^+ channels. Male ICR mice were pre-treated with ATP-sensitive K^+ channel inhibitor glibenclamide (8 mg/kg, *p.o.*) and after 15 min received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Possible involvement of the adenosinergic system. Male ICR mice were pre-treated with theophylline (3 mg/kg, *i.p.*) as a nonselective adenosine receptor antagonist, and after 15 min received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Possible involvement of 5-HT_3-serotonergic receptors. Male ICR mice were pre-treated with the 5-HT_3 receptor antagonist ondansetron (0.5 mg/kg, *i.p.*) and after 15 min received DAE (300 mg/kg, *p.o*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Possible involvement of adrenergic (α_1 & α_2-adrenoceptors). Male ICR mice were pre-treated with prazosin (1 mg/kg, *p.o.*) as a selective α_1-adrenoceptor antagonist, and yohimbine (3 mg/kg, *p.o.*) as a selective α_2-adrenoceptor antagonist). After 15 min, the mice received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Possible involvement of voltage-gated calcium channels. Male ICR mice were pre-treated with nifedipine (10 mg/kg, *p.o.*) as an L-type voltage-gated calcium channel (VGCC) blocker, and after 15 min received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Possible involvement of the muscarinic cholinergic system. Male ICR mice were pre-treated with atropine (5

mg/kg, *i.p.*) as a nonselective muscarinic receptor antagonist, and after 15 min received either DAE (300 mg/kg, *p.o.*), morphine (3 mg/kg, *i.p.*), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 min after morphine administration.

Paclitaxel-induced neuropathic pain

These experiments were carried out as previously described with some modifications [23]. Male Sprague Dawley rats could acclimatize to the behavioural testing environment. Baseline measurements of hot and cold thermal stimuli were performed. Neuropathic pain was induced in the rats by intraperitoneal injection of paclitaxel (2 mg/kg) dissolved in distilled water on four alternate days (days 0, 2, 4 and 6) as described by [23,24]. On day 16 post-paclitaxel treatments, vehicle [10 mL/kg, *p.o.* (group 1)], DAE [100, 300 and 1000 mg/kg, *p.o.* (groups 2-4)] and pregabalin [10, 30 and 100 mg/kg, *i.p.* (groups 5-7)] were administered to the rats after confirmation of neuropathic pain in the various tests. The effect of the vehicle, DAE and pregabalin treatments on paclitaxel-induced neuropathic pain were evaluated in the thermal hyperalgesia and cold allodynia tests.

Thermal hyperalgesia. The hot plate test was used to determine the effect of the vehicle, DAE (100 - 1000 mg/kg) and pregabalin (10 - 100 mg/kg) on thermal hyperalgesia [23]. The rats (pre-treated with the extract or reference drugs as described above) were placed individually on the hot plate maintained at 52 ± 0.5 °C and nociceptive responses (licking/shaking of the paws, jumping) assessed. The latency to pain response was recorded with a cut-off of 20 sec to avoid injury to the animal.

Cold allodynia. The tail-immersion/flick test was used to determine the analgesic effect of the vehicle (10 mL/kg), DAE (100 - 1000 mg/kg) and pregabalin 10 - 100 mg/kg on cold allodynia [25]. This was assessed by immersing the distal portion of the tail (3 - 4 cm) of the rat in cold water maintained at < 4°C until the tail was withdrawn. The duration of immersion was recorded, and a cut-off time of 20 sec was used. Latencies to reaction times pre- and post-drug treatment were measured in the hot plate and tail-flick tests on days 16, 18 and 20 (labelled as Day 0, 3 and 5) post paclitaxel administration. A cut-off reaction time was set at 20 sec to prevent damage to tissues of the foot. The %MPE of individual rats was measured as described above. The AUC was determined graphically and plotted as the total nociceptive score against the dose in mg/kg.

Statistical Analysis

The dose responsible for 50% of the maximal effect (ED_{50}) and statistical analyses were analyzed using GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA). Comparisons with $p < 0.05$ were considered statistically significant. Time-course curves and column graphs were analyzed using two-way and one-way repeated measures ANOVA followed by Bonferroni's/Dunnett's post hoc test, respectively. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

RESULTS

Acetic acid-induced writhing

In this assay, pre-treatment of mice with DAE significantly and dose-dependently reduced the frequency of writhes caused by the intraperitoneal injection of acetic acid (Vehicle, 59.00 ± 5.81; DAE 100 mg/kg, 39.60 ± 2.73; $p = 0.001$, $F_{3,24} = 7.29$] (Figure 1A). Assessment using total writhes, calculated as the AUC also showed that DAE significantly decreased the writhing effects of acetic acid and this was also dose-dependent when compared to the vehicle treated group ($p < 0.001$; $F_{3,16} = 17.80$) (Figure 1B).

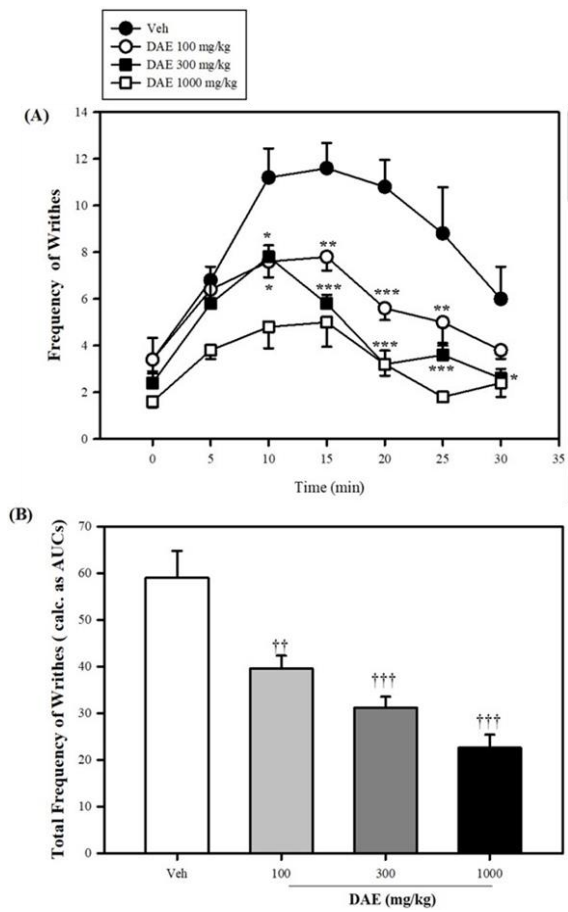

Figure 1: Effect of DAE (100, 300, 1000 mg/kg) on the time course curves (A) and the total nociceptive score (calculated as AUCs) (B) of acetic acid-induced writhing in mice. Nociceptive scores are shown in 5 min time bins up to 30 min for the time course curves. Data presented as Mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group (Veh) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc test). $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ compared to vehicle control group (Vehicle) (one-way ANOVA followed by Dunnet's post hoc test)

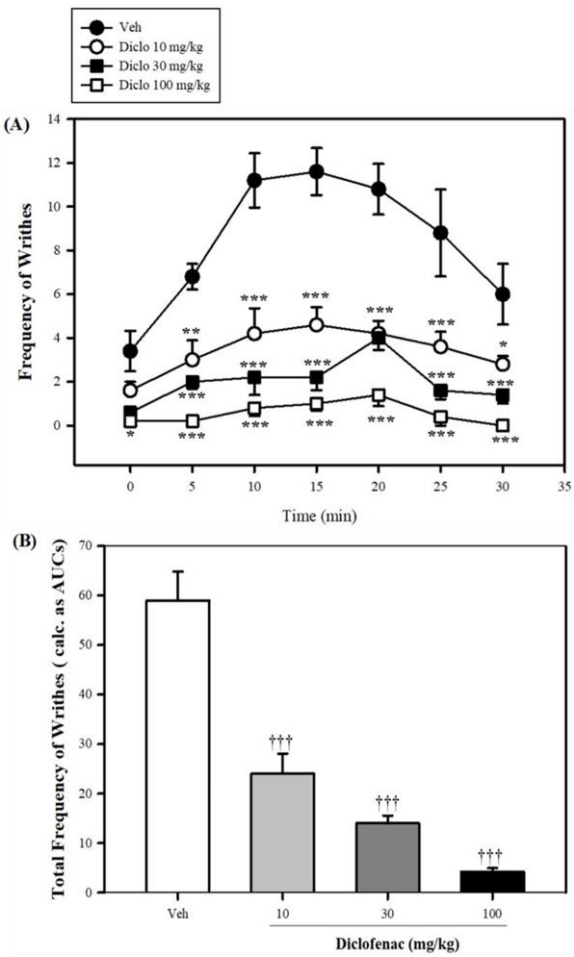

Figure 2: Effect of diclofenac (1, 3, 10 mg/kg) on the time course curves (A) and the total nociceptive score (calc. as AUCs) (B) of acetic acid-induced writhing in mice. Nociceptive scores are shown in 5 min time bins up to 30 min for the time course curves. Data presented as Mean ± SEM. *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$ compared to control group (vehicle) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc test). $^{\dagger\dagger\dagger}P < 0.001$ compared to vehicle control group (vehicle) (one-way ANOVA followed by Dunnet's post hoc test)

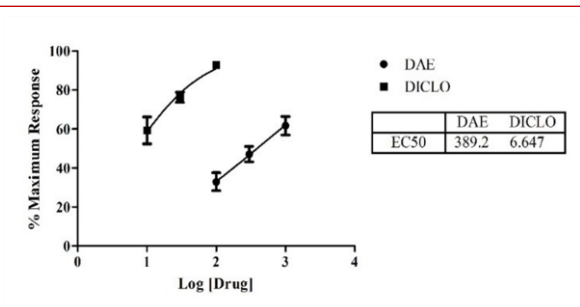

Figure 3: Dose-response curves of DAE (100, 300, 1000 mg/kg) and diclofenac (10, 30, 100 mg/kg) in acetic acid-induced writhing test.

Correspondingly, the non-steroidal anti-inflammatory drug, diclofenac (10 - 100 mg/kg) also significantly and dose-dependently attenuated the frequency of writhes induced by acetic acid ($p < 0.001$, $F_{3,24} = 26.88$) (Figure 2A). Calculated AUCs also revealed significant inhibition of writhing in comparison to the vehicle-treated groups and this inhibition of writhes was dose-dependent (Vehicle, 59.00 ± 5.805; Diclofenac 10 mg/kg, 24.00 ± 4.07; $p < 0.001$, $F_{3,16} = 42.83$) (Figure 2B). From the ED values obtained by log-dose graph (Figure 3), diclofenac was about 60 times more potent than DAE.

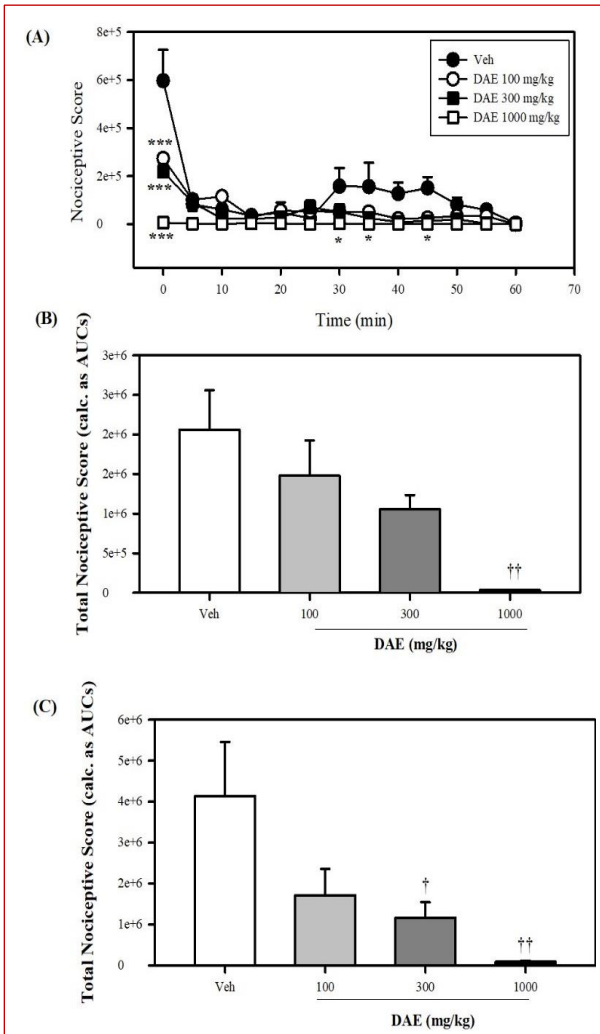

Figure 4: Effect of DAE (100, 300, 1000 mg/kg) (A) and the total nociceptive score (calc. as AUCs) (B, C) in the 1st and 2nd phases of formalin-induced nociception in mice. Nociceptive scores are shown in 5 min time bins up to 60 min for the time course curves. Data are presented as mean ± standard error (n = 5). *$p < 0.05$, ***$p < 0.001$ compared to control group (vehicle) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc test). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared to control group (vehicle) (one-way ANOVA followed by Dunnet post hoc test)

Formalin-induced licking

In the DAE treated group, there was a general reduction in pain as described by nociceptive scores and this reduction was dose-dependently significant for all the doses of DAE (100 - 1000 mg/kg) used in comparison to the vehicle (p = 0.011, $F_{3,48}$ = 4.12) (Figure 4A). Oral administration of DAE (100, 300, 1000 mg/kg) 30 min before formalin injection dose-dependently inhibited both the first and second phases of formalin-induced paw biting/licking but this inhibition was only significant for DAE (1000 mg/kg) during the first phase (p = 0.006, $F_{3,16}$ = 6.09; Figure 4B) and DAE (300 -1000 mg/kg) during the second phase (p = 0.012, $F_{3,16}$ = 5.09; Figure 4C). Similarly, morphine (1, 3, 10 mg/kg) which is an opioid agonist significantly and dose-dependently reduced nociceptive scores in comparison to the vehicle (p = 0.002; $F_{3,48}$ = 4.12; Figure 5A). Also, when nociceptive scores were calculated as AUCs, morphine (1, 3, 10 mg/kg) significantly and dose-dependently inhibited both the first and second phases of formalin-induced nociceptive behaviours (p = 0.001; $F_{3,16}$ = 13.29 and p = 0.013; $F_{3,16}$ = 5.011, respectively) (Figures 5B and 5C). Furthermore, diclofenac (10, 300, 100 mg/kg), a non-steroidal anti-inflammatory drug, significantly and dose-dependently reduced total nociceptive scores induced by formalin injection when compared to the vehicle. (p = 0.004; $F_{3,48}$ = 3.08, Figure 6A). Diclofenac reduced nociceptive response during the first phase, but this reduction was only significant at the highest tested dose of

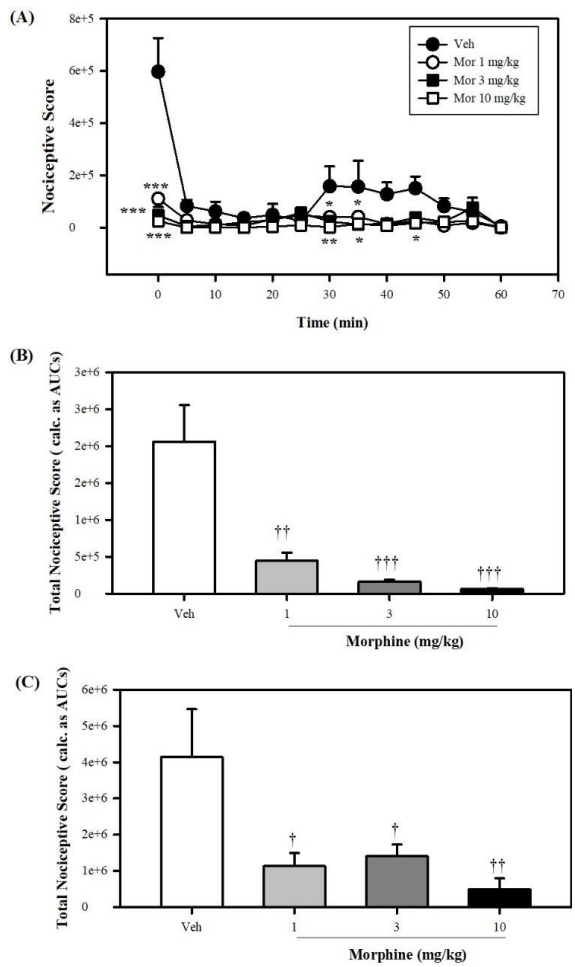

Figure 5: Effect of morphine (1, 3, 10 mg/kg) (A) and the total nociceptive score (calculated as AUCs) (B, C) in the 1st and 2nd phases of formalin-induced nociception in mice. Nociceptive scores are shown in 5 min time bins up to 60 min for the time course curves. Data are presented as mean ± standard error (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group (vehicle) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc test). †p < 0.05, ††p < 0.01, †††p < 0.001 compared to control group (ctrl) (one-way ANOVA followed by Dunnet post hoc test)

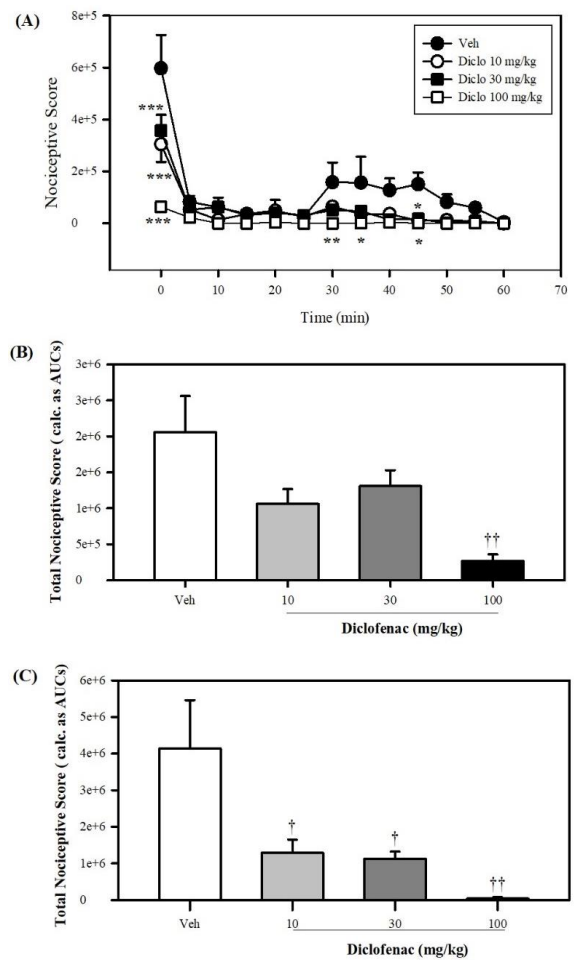

Figure 6: Effect of diclofenac (10 - 100 mg/kg) (A) and the total nociceptive score (calculated as AUCs) (B, C) in the 1st and 2nd phases of formalin-induced nociception in mice. Nociceptive scores are shown in 5 min time bins up to 60 min for the time course curves. Data are presented as mean ± standard error (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group (Veh) (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc*). †p < 0.05, ††p < 0.01 compared to control group (Veh) (one-way ANOVA followed by Dunnet post hoc test)

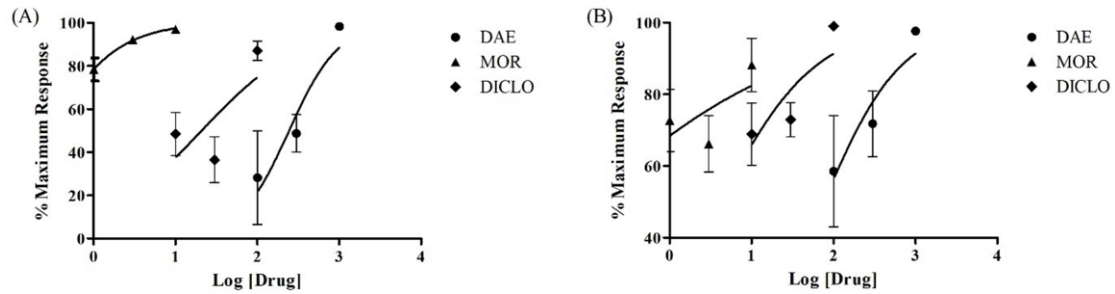

Figure 7: Dose-response curves of DAE (100, 300, 1000 mg/kg), morphine (1, 3, 10 mg/kg) and diclofenac (10, 30, 100 mg/kg) in the 1st and 2nd phases of formalin induced nociception test.

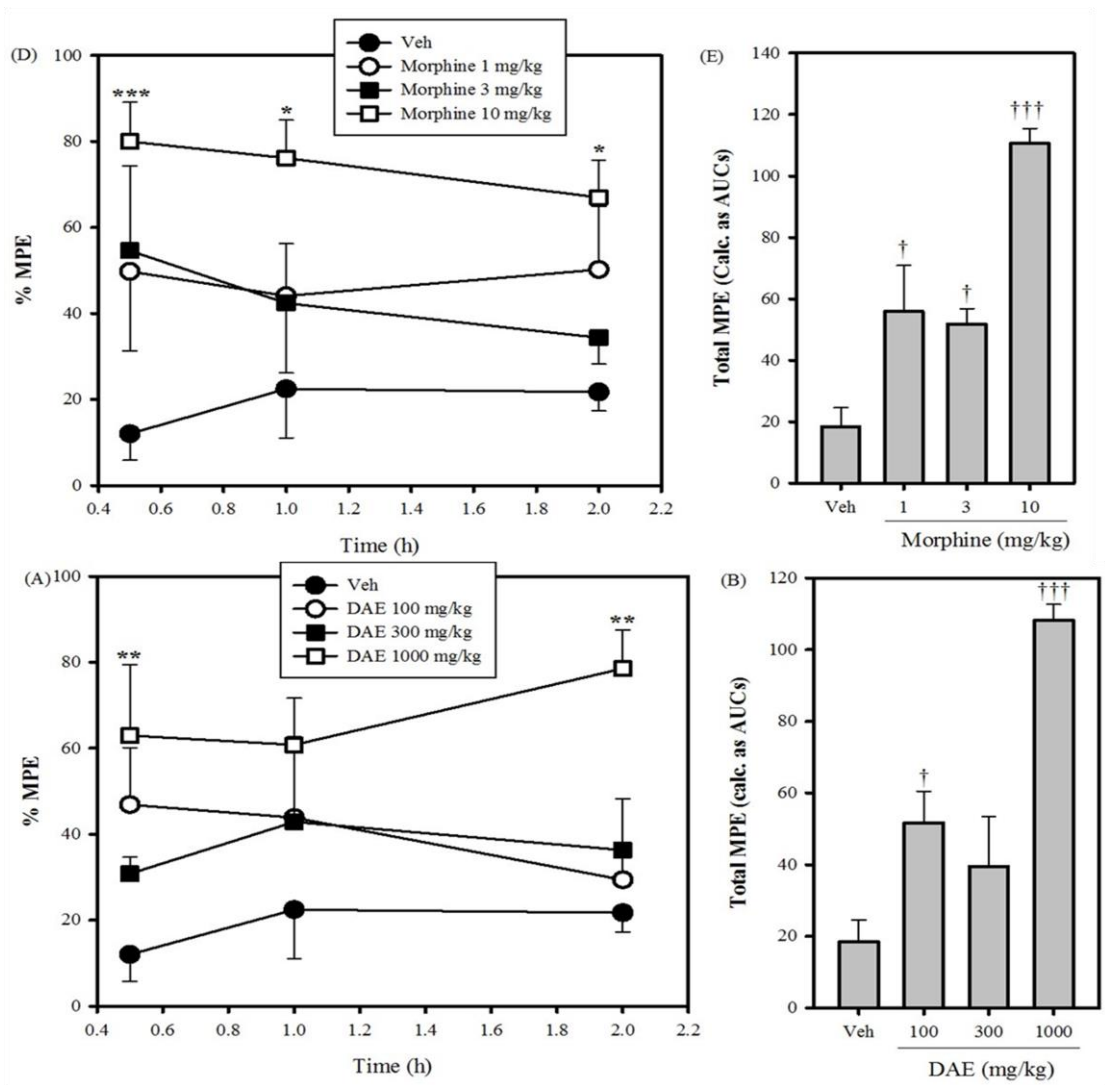

Figure 8: Effect of DAE (100-1000 mg/kg, *p.o.*) and vehicle (Veh) on the assessment of analgesia using % MPE and AUC in the hot plate test. Data are presented as mean ± standard error (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group (Veh) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc). †$p < 0.05$, ††$p < 0.01$compared to control group (Veh) (one-way ANOVA followed by Dunnet post hoc test)

diclofenac (100 mg/kg) (p = 0.005, $F_{3,16}$ = 6.280; Figure 6B). However, during the second phase, diclofenac (10, 30, 100 mg/kg) significantly and dose-dependently reduced nociceptive responses induced by formalin (p = 0.012, $F_{3,16}$ = 6.41; Figure 6C). Comparison of ED_{50} obtained from a log-dose response curve indicated a sigmoid displayed dose-response relationship as shown in Figure 7. The ED_{50} values were approximately 244.00, 0.28, 20.83 mg/kg for the first phase and 75.85, 0.10, 4.09 mg/kg for the second phase. Comparison of ED_{50} values obtained showed that the extract was more potent in the second phase than in the first. The graph also indicated that morphine was the most potent followed by diclofenac and then DAE in both first and second phases of formalin-induced nociceptive behaviours. Morphine and diclofenac were approximately 870-fold and 12-fold more potent than DAE during the first phase (Figure 7A), and approximately 770-fold and 20-fold more potent than DAE during the second phase, respectively (Figure 7B).

Assessment of analgesia using the hot plate test

There were differences between the vehicle and the DAE treatment groups on the % MPE. However, this difference was not statistically significant except at a dose of 1000 mg/kg ($p < 0.001$, $F_{3,8}$ = 19.32; Figure 8A). When MPE was calculated as AUC, significant differences were noted between the vehicle and the DAE treatment groups at doses of 100 mg/kg and 1000 mg/kg (vehicle, 18.43 ± 6.15 vs DAE 100 mg/kg, 51.69 ± 8.79; $p < 0.001$, $F_{3,10}$ = 24.95) (Figure 8B) as shown by the post hoc test. There was also no statistically significant difference between the vehicle and the morphine treatment groups on %MPE except at dose 10 mg/kg ($p < 0.001$, $F_{3,8}$ = 32.08; Figure 9D).

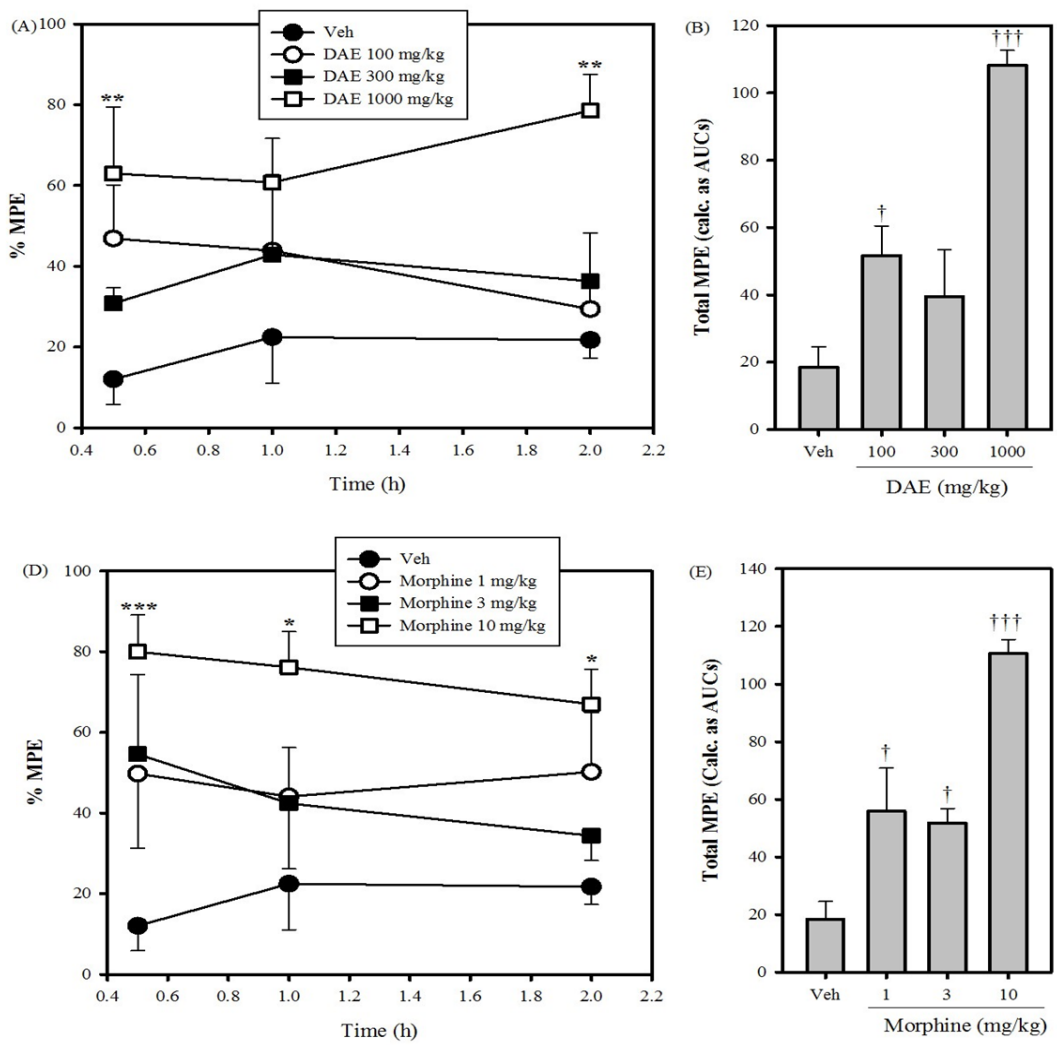

Figure 9: Effect of Morphine (1, 3, 10 mg/kg, *i.p.*) and vehicle (Veh) on the assessment of analgesia using % MPE and AUC in the hot plate test. Data are presented as mean ± standard error (n=5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group (Veh) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc). $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared to control group (Veh) (one-way ANOVA followed by Dunnet post hoc test)

However, when %MPE was calculated as AUC there was a significant difference noted between the vehicle and the morphine treatment groups at all doses used (1 - 10 mg/kg) and more pronounced at 10 mg/kg as revealed by the post hoc test (vehicle,18.43 ± 6.15; morphine 10 mg/kg, 55.88 ± 14.95; $p < 0.001$, $F_{3,10} = 22.16$) (Figure 9E) suggesting that AUC assessment is better at detecting changes in this behaviour in mice.

Mechanism(s) of action to DAE on pain. The results presented in Figure 10 show that the pre-treatment of mice with naloxone (2 mg/kg, *i.p.*) significantly ($p < 0.001$) inhibited antinociception by DAE (300 mg/kg, *p.o.*) in the hot plate test. Naloxone also significantly ($p < 0.001$) reversed the antinociception caused by morphine (3 mg/kg, *i.p.*) (Figure 10). Systemic pre-treatment of mice with glibenclamide (8 mg/kg, *i.p.*) significantly ($p < 0.001$) reduced the antinociception caused by DAE (300 mg/kg, *p.o.*) (Figure 10) but not the effect of morphine ($p > 0.050$). Systemic oral pre-treatment of mice with prazosin (1 mg/kg *p.o*) significantly ($p < 0.001$) reduced antinociceptive action of DAE (300 mg/kg, *p.o.*) in the test (Figure 10). It also reversed ($p < 0.005$) the antinociceptive effect of morphine (3 mg/kg, *i.p.*) (Figure 10). Yohimbine (3 mg/kg, *i.p.*) also significantly ($p < 0.001$) inhibited antinociception of DAE (300 mg/kg, *p.o.*; Figure 10) as well as morphine (3 mg/kg, *i.p.*; Figure 10). Theophylline and ondansetron (0.5 mg/kg, *i.p.*) both significantly reduced antinociceptive activity caused by morphine (3 mg/kg, *i.p.*) ($p < 0.001$ and $p < 0.050$, respectively; Figure 10) but did not affect antinociception produced by DAE (300 mg/kg, *p.o*). L-NAME (10 mg/kg, *i.p.*), atropine (5 mg/kg, *i.p.*) and nifedipine (10 mg/kg, *p.o.*) all did not significantly inhibit the antinociception caused by either DAE (300 mg/kg, *p.o.*) or morphine (3 mg/kg, *i.p.*) in the hot plate test.

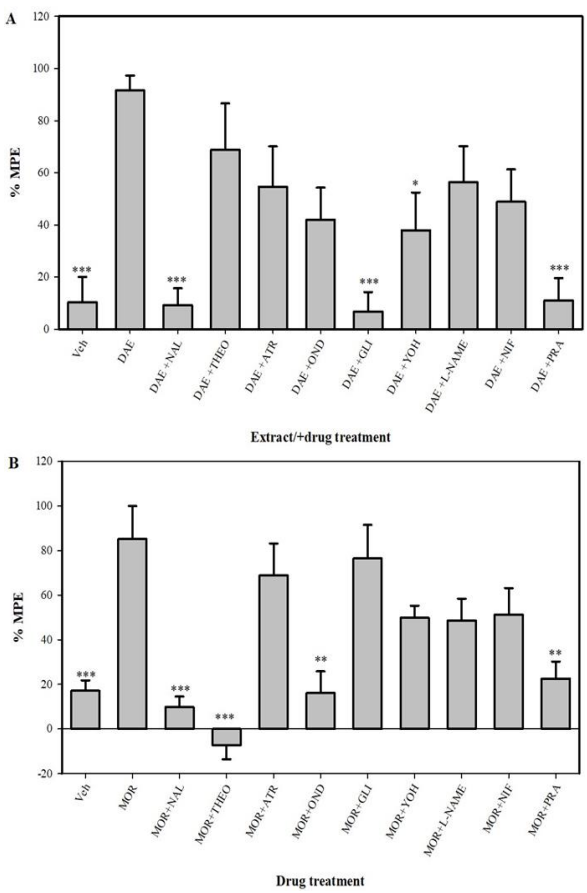

Figure 10: Effect of pretreatment of mice with naloxone (NAL) (2 mg/kg, *i.p.*), theophylline (THEO) (10 mg/kg, *i.p.*), L-NAME (10 mg/kg, *i.p.*), glibenclamide (GLI) (8 mg/ kg, *p.o.*), ondansetron (OND) (0.5 mg/kg, *i.p.*), atropine (ATR) (5 mg/kg, i.p.), yohimbine (YOH) (3 mg/kg, *p.o.*), and nifedipine (NIF) (10 mg/kg, *p.o.*) and prazosin (PRA) (1 mg/kg) on the percent maximal effect of (A) DAE 300 mg/kg, *p.o.* and (B) Morphine (MOR) 3 mg/kg, *i.p*, in the hot plate test. Data are presented as mean ± standard error (n=5). *$p \leq 0.05$, ***$p \leq 0.001$ compared with DAE/MOR only treated group respectively (one-way ANOVA followed by a Dunnett's multiple comparison post hoc test)

Effect of DAE and other analgesics on paclitaxel-induced neuropathic pain.

Thermal hyperalgesia. Neuropathic pain was confirmed in thermal hyperalgesia models on the day 16 post paclitaxel injection. The DAE (100 – 1000 mg/kg) produced significant analgesic properties from day 1 to 5 ($p < 0.001$; $F_{3,64} = 10.95$) (Figure 11A) when nociceptive scores were measured as % MPE on alternate days. Furthermore, when nociceptive scores were measured as AUC, there was a significant difference between DAE (100, 300, 1000 mg/kg) and the vehicle, with all the doses significantly reversing thermal hyperalgesia in the model used (vehicle, 168.7 ± 13.68; DAE 100 mg/kg, 54.81 ± 7.65); $p = 0.016$, $F_{3,12} = 9.70$) (Figure 11B). Pregabalin (10, 30, 100 mg/kg) similarly produced analgesic properties in this model ($p < 0.001$, $F_{3,64} = 16.11$) (Figure 11C). Pregabalin (10, 30, 100 mg/kg) also significantly produced similar analgesic properties when nociceptive scores was measured as AUC (vehicle, 168.70 ± 13.68; pregabalin 10 mg/kg, 53.64 ± 18.62; $p = 0.004$, $F_{3,12} = 7.7$) (Figure 11D).

Cold allodynia. There was no significant difference between the vehicle and the treatment groups except for DAE at dose of 100 mg/kg and 300 mg/kg. DAE at these doses significantly reversed cold allodynia only on the 5th day of allodynia ($p = 0.002$, $F_{3,64}=5.36$) (Figure 12A). Furthermore, when nociceptive score was measured as AUC, only DAE at 300 mg/kg significantly reversed cold allodynia induced (vehicle, 139.00 ± 39.48; DAE 100 mg/kg, 99.71 ± 37.61; $p = 0.075$, $F_{3,12} = 2.9$) (Figure 12B). In the pregabalin treated group, a dose of 30 mg/kg only reversed cold allodynia on the 5th day but pregabalin at 100 mg/kg significantly reversed cold allodynia from day 1 to 5 ($p < 0.001$, $F_{3,64} = 17.45$) (Figure 12C) in the model. However, when nociceptive scores were measured as AUC, pregabalin (10, 30, 100 mg/kg) significantly reversed cold

allodynia in the model used (vehicle, 139.00 ± 39.48; pregabalin 10 mg/kg, 106.80 ± 35.83; $p = 0.002$, $F_{3,12} = 8.9$) (Figure 12D).

DISCUSSION

The findings of the present study indicated that the ethanolic extract of *D. adscendens* acts on the central nervous system and possesses analgesic, anti-nociceptive and anti-neuropathic pain activities. The acetic acid-induced abdominal writhing model in mice is reliable, easy to perform, sensitive and particularly suitable for evaluating even weak analgesics [26]. The peritoneum has silent nociceptors attached to C-polymodal fibres which are only activated in inflammation. Thus, intraperitoneal administration of acetic acid triggers the synthesis and/ or release of prostaglandins which subsequently cause the production of bradykinin, a noxious endogenous substance within the peritoneum [27,28]. Furthermore, intraperitoneal administration of acetic acid induces a characteristic and quantifiable overt pain-like behaviour described as a writhing response or abdominal contortions, characterized by abdominal distention and outstretching of hind limbs [29]. Related studies have also implicated interleukins (IL-1β, IL-8) and tumour necrosis factor-alpha (TNF-α) in mast cells and resident macrophages within the peritoneum [30]. The expression of these substances produces spontaneous dorso-abdominal muscle contraction. The antinociceptive effect of DAE against the acetic acid-induced nociception, suggests that DAE may inhibit the release and or synthesis of inflammatory mediators, pro-inflammatory cytokine, partial or complete blockage of these silent nociceptors present in the peritoneum. These results confirm the findings by N'gouemo et al. (1996) on the inhibition of writhing by DAE in the acetic-acid test [15]. The formalin test is specific and particularly useful for the assessment of new analgesics since it encompasses neurogenic and inflammatory mechanisms of nociception [31,32]. Opioids are known to inhibit both phases whereas non-steroidal anti-inflammatory drugs mostly affect the inflammatory phase [26]. The extract, DAE, exhibited an obvious anti-nociceptive activity in all phases of the test. This suggests that DAE may have a direct effect on nociceptors associated with the early phase of the test. Additionally, the inhibition of pain in the second phase may be due to a modulatory effect on the release and/or synthesis of pro-inflammatory mediators [33] or subsequent cerebrospinal processing of pain after activation of the nociceptors.

To further investigate the involvement of central pain pathways in the analgesic effect of DAE, hot plate test was used. The significant increase in latency time by DAE in the hot plate method suggests the involvement of central mechanisms [34] in the anti-nociceptive activity of DAE. The extract may have constituents that interact with nerve endings and/or inhibit the release of neurotransmitters and peptides from both peripheral and central nerve terminals. Thus reduce pain generation and perception or by activating the descending inhibitory pathway of pain [35,36]. To evaluate the possible mechanism(s) of action of DAE, the analgesic effect of DAE was assessed in the presence of various antagonists of known mediators in the pain pathway

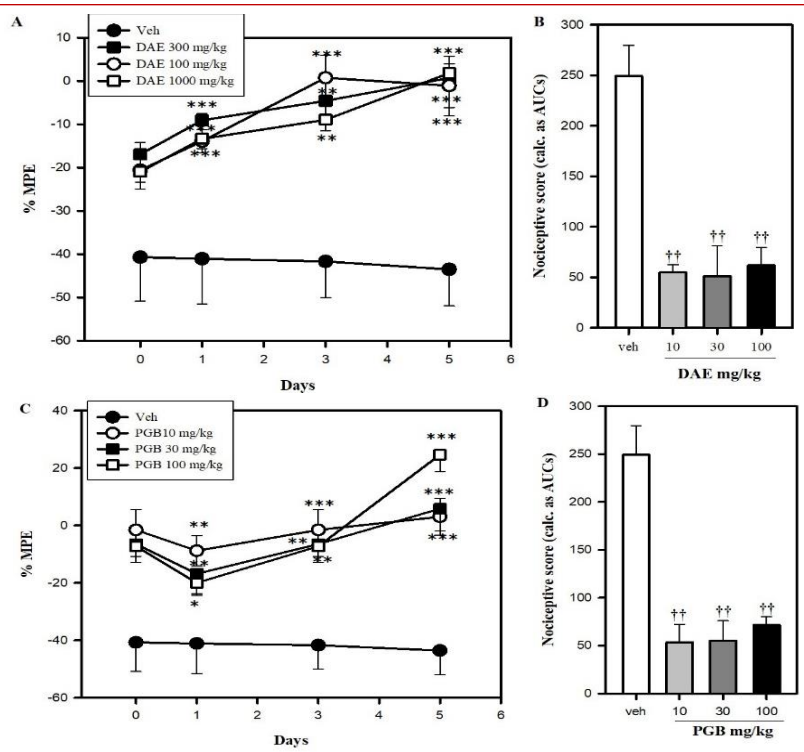

Figure 11: The effects of daily dosing of DAE (100 - 1000 mg/kg) pregabalin (PGB 10 - 100 mg/kg) and vehicle on established paclitaxel-induced thermal hyperalgesia as a time-course curve in the hot plate test and nociceptive scores measured as AUC. Graph A and C represent time-course curve of DAE and PGB respectively whiles graph B and D represent AUC of DAE and PGB respectively. Data are presented as mean ± standard error (n = 5). *$p <$ 0.05, **$p <$ 0.01, ***$p <$ 0.001 compared to control group (Veh) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc). †$p <$ 0.05, ††$p <$ 0.01 compared to control group (Veh) (one-way ANOVA followed by Dunnet post hoc)

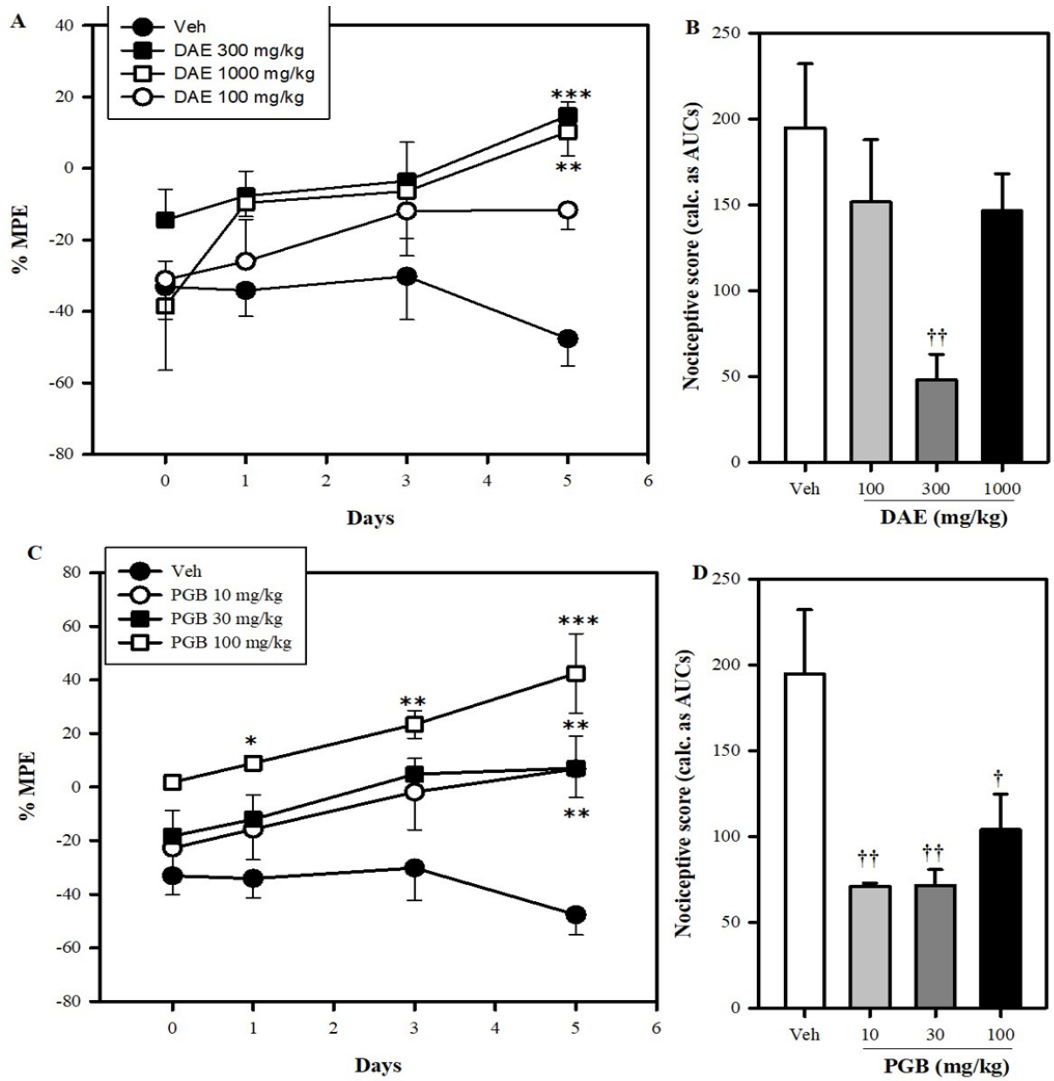

Figure 12: The percent maximal effects of daily dosing of DAE (100 - 1000 mg/kg), pregabalin (10 - 100 mg/kg) and vehicle on established paclitaxel-induced cold allodynia as a time-course curve, and nociceptive scores measured as AUC. Graphs A and C represent time-course curve of DAE and PGB respectively, while graphs B and D represent AUC of DAE and PGB respectively. Data are presented as mean ± standard error (n=5). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control group (Veh) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc). $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared to control group (Veh) (one-way ANOVA followed by Dunnet post hoc test)

including naloxone, theophylline, L-NAME, glibenclamide, atropine, ondansetron, yohimbine, prazosin and nifedipine. The hot plate test was used because the extract showed pronounced activity in this test. Naloxone, a nonselective opioid antagonist, significantly reversed the analgesic effect of DAE in the test, signifying a possible opioidergic involvement in the actions of DAE. This strongly suggests DAE's analgesic effect is via interaction at or with opioid receptors at the supra-spinal level, consistent with the Straub tail effect first observed in Irwin's test. In agreement with this suggestion, it has been demonstrated that μ-opioid receptors may mediate mainly supra-spinal analgesia [37]. This result suggests that DAE may have a prominent effect on central μ-opioid receptors. This finding suggests that the activation of opioid receptors and/or an increase in endogenous opioids, either centrally or peripherally [38] might be involved in the analgesic effect of DAE [39]. Glibenclamide is an ATP-sensitive K^+ channel blocker which blocked the analgesic activity of both DAE and morphine. The opening of ATP-sensitive K^+ channel has been reported to participate in opioid-mediated antinociception at the level of K^+ and not opioid receptor activation [40-42]. It is well established that glibenclamide specifically blocks ATP sensitive K^+ channels, with no effect on Ca^{2+} or voltage-dependent K^+ - channels [43]. The present data suggest that the opening of ATP-sensitive

K^+ channels plays a significant role in the analgesic action of DAE. This pharmacological action of DAE has been evaluated and described by several researchers as a potent K^+ channel opener [44-46]. Neurotransmitter release from neurons is normally preceded by depolarization of the nerve terminal and Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. Drugs may inhibit neurotransmitter release by a direct effect on Ca^{2+} channels to reduce Ca^{2+} entry, or indirectly by increasing the outward K^+ current, thus shortening repolarisation time and the duration of the action potential [48]. The latter may be the possible case in the analgesic effect of DAE since its effects were reversed by glibenclamide but not nifedipine. Opioidergic drugs produce both effects because opioid receptors are coupled via G-proteins directly to K^+ channels. It is likely that compounds that open K^+ channels may gain importance as effective pain relievers since these are very effective in models of acute and chronic pain [47].

The noradrenergic receptor system is greatly involved in descending modulation of pain pathways. Clonidine, an α_2 adrenergic agonist, acting on the nerve endings of primary afferent fibres can inhibit the release of norepinephrine, glutamate and substance P, as well as proinflammatory cytokines resulting in sedative and analgesic actions [48, 49]. The current findings suggest and corroborate an earlier study of the possible involvement of adrenergic system [44-46,50] since DAE activity was significantly reversed when pretreated with yohimbine (α_2-adrenergic receptor antagonist) and prazosin (α_1-adrenergic receptor antagonist) in this study. Also, serotonergic pathways interact with the noradrenergic system. Activation of serotonergic receptors can cause the release of noradrenaline which can activate postsynaptic α_2-adrenergic receptors in the spinal cord leading to anti-nociception [51,52]. Pre-treatment with yohimbine (which also inhibits 5-HT_{2A} receptor) significantly reduced DAE's analgesic activity indicating its possible utilization of the serotonergic system to reduce pain. However, the analgesic effect of DAE was not significantly affected in the presence of ondansetron, a $5HT_3$ receptor antagonists, implying that a particular serotonin pathway utilizing $5HT_{2A}$ receptors but not $5HT_3$ receptors [53] is involved in DAE's effects. Since the analgesic effect of DAE was not significantly affected by theophylline (an adenosine receptor antagonist), atropine (a muscarinic receptor antagonist), nifedipine (L-type voltage-gated Ca^{2+} channel blocker), L-NAME (nitric oxide synthase inhibitor) and ondansetron ($5HT_3$ receptor antagonist), it is suggested that DAE may not, or to a lesser extent, exert its analgesic activity via these pathways.

Chemotherapy-induced peripheral neuropathy (CIPN) is a common disturbing adverse effect in the use of certain anticancer agents that have led to poor compliance and in some cases the discontinuation of chemotherapy [54]. Agents, such as paclitaxel, are a major culprit. Paclitaxel, a widely used chemotherapeutic agent for the treatment of solid tumours, [55], pharmacokinetically distributes in the central and peripheral nervous system in rats following its administration [56] and accumulates mainly in the dorsal root ganglia and the brain at very low concentrations. Accumulation has also been reported in the sciatic nerve and spinal cord at higher concentrations [56]. Though the precise mechanism by which paclitaxel causes peripheral neuropathy is yet to be established, current studies report that continuous administration of paclitaxel causes severe peripheral neuropathy characterized by thermal and mechanical hyperalgesia as well as allodynia. It does this possibly due to atypical (swollen and vacuolated) mitochondria in peripheral sensory axons of both the C-fibre and myelinated axons, nerve damage by disruptive formation of microtubules needed for axonal transport in the dorsal root ganglia, axons and Schwann cells. A loss of intra-epidermal nerve fibres leading subsequently to loss of cellular function may also be a possible mechanism [55]. In the present study, DAE significantly attenuated paclitaxel-induced hyperalgesia as evidenced by increased tail withdrawal and reaction latencies in the tail-flick and hot plate tests when compared with vehicle and paclitaxel treated group and this was comparable to the effect produced by pregabalin. Pregabalin is the drug of choice for the pharmacological management of CIPN [57, 58].

The analgesic and anti-epileptic actions of pregabalin are associated with its antagonistic effect on α2–δ1 subunit of N-type voltage-dependent calcium channels [58]. Studies have shown that inhibition of calcium channels significantly reduces neuronal excitability by attenuating neuronal calcium influx, thereby causing the inhibition of the release of neurotransmitters including noradrenaline, substance P and glutamate. The inhibition of synaptic transmission and other cellular enzymatic cascade reactions can lead to pain sensation. Also, opioids such as morphine that act via the opioidergic nociceptive pathway have been shown to inhibit paclitaxel-induced neuropathic pain [59]. DAE has been known to suppress pain via the opioidergic nociceptive pathway and this may partly contribute to the anti-neuropathic pain properties of DAE in this model. Several reports indicate that paclitaxel causes the release of pro-inflammatory pain mediators and cytokines, including bradykinin and TNF-α as well as the activation of microglial and astroglial cells. Therefore, the effect of DAE on pro-inflammatory pain mediators and cytokines as a possible mechanism cannot be ruled out and need to be further investigated. These mechanisms and possibly others may be responsible for the highly significant DAE-induced attenuation of pain caused by paclitaxel in this neuropathic pain model.

Conclusion

The ethanolic extract of *D. adscendens* possesses central and peripheral anti-nociception effects and may do this possibly via the interaction with opioid receptors, ATP-sensitive K^+ channels and the adrenergic system. This may suggest the utility of the extract in the management of inflammatory, neurogenic and/or muscle pain states. The study also provides pharmacological evidence to the folkloric use of the plant as an analgesic.

DECLARATIONS

Ethical considerations

Study approval was obtained from the Noguchi Memorial Institute for Medical Research-Institutional Animal Care and Use Committee (IACUC), College of Health Sciences, the University of Ghana with protocol number NIACUC-2017-06-2R. All animal procedures and techniques used in these studies were following IACUC.

Consent to publish

All authors consented to the publication of the manuscript.

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Competing Interests

No conflict of interest was reported by the authors.

Author contributions

ASB, PA, KAB, KEK and SBK participated sufficiently in the intellectual content, conception, and design of this work. ASB, JAL, SA, DOS and PA performed the experiments, analysis, and interpretation of data obtained. ASB & PA drafted the initial manuscript and all authors made inputs and corrections. All authors read and made corrections to the finalized manuscript before submission.

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Availability of data

All data generated or analysed during this study are included in this published article. However, the datasets used and/or analysed during the current study are also available from the corresponding author on reasonable request.

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