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Further studies on the anti-nociceptive and anti-inflammatory effects of the aerial parts of *Hilleria latifolia* (Lam.) H. Walt.

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Abstract

The aerial parts of *Hilleria latifolia* are used in Ghanaian traditional medicine for the treatment of pain and inflammatory disorders. In the current study, the anti-nociceptive and anti-inflammatory effects of the hydro-ethanol (HAE), ethyl acetate (EAE) and petroleum ether (PEE) extracts of the aerial parts of the plant were investigated in animal models. The analgesic effects were assessed in the acetic acid-induced writhing and formalin tests while the anti-inflammatory activities were tested in the carrageenan-induced oedema model in chicks. HAE (10-100 mg kg-1, p.o.), EAE (10-100 mg kg-1, p.o.), PEE (10-100 mg kg-1, p.o.), together with morphine (1-10 mg kg-1, p.o.) and diclofenac (10-100 mg kg-1, p.o.) (positive controls), showed significant anti-nociceptive activity in all the models used. The anti-nociceptive effect exhibited by HAE (30 mg kg-1, p.o.) and PEE (100 mg kg-1, p.o.) were significantly inhibited in the formalin test by the systemic administration of theophylline (10 mg kg-1, i.p). HAE (10-100 mg kg-1, p.o.), given pre-emptively or curatively, significantly inhibited carrageenan-induced foot oedema in 7-day old chicks. PEE (10-100 mg kg-1, p.o.) also significantly attenuated oedema in chicks on curative treatment. In conclusion, the results indicate that HAE, EAE and PEE produce dose-related analgesic effects in mice. The mechanism of action of HAE and PEE involve an interaction with the adenosinergic system. Also, HAE and PEE have significant anti-inflammatory properties after oral administration in animals.

Keywords: *Hilleria latifolia*, Formalin test, Writhing test, Theophylline, Carrageenan, Chicks.

Introduction

Hilleria latifolia (Lam.) H. Walt. (Phytolaccaceae) is a perennial herb that is common on cultivated grounds and along forest paths in the forest regions of Ghana.[1] It also occurs in other parts of tropical Africa as well as South America. It is commonly known as a*nafranaku* by the Akans and a*vegboma*, *boe* or *kukluigbe* by the Ewes.[1] Various parts of the plant are used in Ghanaian traditional medicine for the treatment of pain and inflammatory diseases. The leaves are effective in otalgia [2], rheumatism [1, 2] and boils [1] whereas the flowers are used for asthma.[2]

Phytochemical tests carried out on the crude extract of the aerial parts of the plant revealed the presence of saponins, tannins, glycosides, steroids, terpenoids as well as little amounts of flavonoids and alkaloids.[3] The ethanol (70 %) extract of the aerial parts of *Hilleria latifolia* has been reported to produce dose-related anti-nociception in several models of chemical and thermal pain, without induction of tolerance, through mechanisms that involve an interaction with adenosinergic, muscarinic cholinergic and opioid pathways.[3] Also, the ethanol (70 %) extract exerts in vivo anti-inflammatory activity after oral administration and has antioxidant properties which may contribute to its activity.[4] The anxiolytic- and antidepressant-like properties have also been established.[5]

The present study is a follow-up on our earlier works [3, 4] in which we showed that the crude extract of the aerial parts of *Hilleria latifolia* has anti-nociceptive and anti-inflammatory effects in animals. As part of ongoing efforts to identify the active constituents responsible

for the analgesic and anti-inflammatory properties, we have serially extracted the aerial parts of the plant with petroleum ether, ethyl acetate and 70 % ethanol to obtain three fractions. We report in this study the anti-nociceptive and anti-inflammatory effects of the three extracts (fractions).

Materials and Methods

Plant materials

The aerial parts of *H. latifolia* were collected from the campus of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi near the Botanical Gardens (06°41′12.89″N; 01°33′59.51″W) during the month of September, 2012 and authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/HM1/09/L029) was kept at the herbarium of the Faculty.

Preparation of extracts

The aerial parts were room-dried for about two weeks and pulverised into fine powder. Three extracts were obtained from the powder by successive cold percolation with petroleum ether, ethyl acetate and 70 % (v/v) ethanol respectively. The resulting extracts were each concentrated into a syrupy mass under reduced temperature (60 ˚C for petroleum ether and ethyl acetate extracts and 70 ˚C for the ethanol extract) and pressure using a rotary evaporator. The extracts were further dried over a water bath and then kept in a desiccator for use. The yields were 1.97 % $^{w}/_{w}$ (petroleum ether extract, PEE), 3.09 % $^{w}/_{w}$ (ethyl acetate extract, EAE) and 18.09 % $^{w}/_{w}$ (hydro-ethanol extract, HAE).

Drugs and chemicals

The following drugs and chemicals were used: acetic acid, formalin and theophylline (British Drug Houses Ltd, Poole, England); diclofenac (KRKA®, Novo Mesto, Slovenia); morphine (PhytoRiker®, Accra, Ghana) and λ-carrageenan (Sigma-Aldich Inc., St. Louis, MO, USA). The extracts were suspended in 2 % tragacanth.

Animals

Cockerels (*Gallus gallus*; strain Shaver 579, Akropong Farms, Kumasi, Ghana) were obtained one day post-hatch and housed in stainless steel cages (34 cm × 57cm × 40 cm) at a population density of 12–13 chicks per cage. Food (Chick Mash, GAFCO, Tema, Ghana) and water were available *ad libitum* through 1-qt gravity-fed feeders and waterers. Overhead incandescent illumination was provided with room temperature at 29 °C. Chicks were tested at 7 days of age.

Male ICR mice (25±5 g) were purchased from Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept at the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, (KNUST), Kumasi. They were housed in groups of 5 in stainless steel cages (34×47×18 cm^3) with soft wood shavings as bedding, fed with solid pellet diet (GAFCO, Tema, Ghana), given water *ad libitum* and maintained under laboratory conditions (temperature 24±2 °C, relative humidity 60-70 %, and 12 hour light-dark cycle). All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85 - 23, 1985, revised 1996). All protocols used were approved by the Departmental Ethics Committee.

Phytochemical screening

Preliminary phytochemical tests were performed on the extracts using methods described by Trease and Evans.[6]

Acetic acid-induced writhing test

The test was performed as described earlier by Woode and Abotsi.[3] Briefly, male mice were pre-treated with the test preparations [HAE, EAE or PEE (10, 30 and 100 mg kg^{-1}, *p.o.*); diclofenac (10, 30 or 100 mg kg^{-1}, *p.o.*) or vehicle (10 ml kg^{-1}, *p.o.*)] 1 h before administration of acetic acid 0.6 % (10 ml kg^{-1}, i.p.). Total number of abdominal writhes per 5 min was determined for 25 minutes after acetic acid administration. Data obtained for the first 5 minutes after acetic acid administration was ignored in the data analysis.

Formalin-induced nociception

The formalin test, first described by Dubuisson and Dennis [7], was carried out as modified by Woode and Abotsi.[3] Male mice were pre-treated with the test preparations [HAE (30 mg kg^{-1}, *p.o.*), EAE (100 mg kg^{-1}, *p.o.*), PEE (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 ml kg^{-1}, *p.o.*)] 30 min (i.p.) or 1 h (*p.o.*) before the intraplantar injection of 10 µl of 5 % formalin. Pain response (biting/licking) 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. The average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking. Data were expressed as the mean ± SEM of scores between 0–10 min (first phase) and 10–60 min (second phase) after formalin injection.

To determine the involvement of the adenosinergic system in the action of the extracts, mice were pre-treated with theophylline (10 mg kg^{-1}, i.p, a non-selective adenosine receptor antagonist). After 15 min, the mice received HAE (30 mg kg^{-1}, *p.o.*), EAE (100 mg kg^{-1}, *p.o.*), PEE (100 mg kg^{-1}, *p.o.*) or vehicle (10 ml kg^{-1}, *p.o.*). The nociceptive response to intraplantar injection of formalin was recorded 60 min after administration of extracts or vehicle.

Carrageenan-induced Oedema in Chicks

The anti-inflammatory activity of the extracts were assessed using the carrageenan-induced foot oedema model in the chick [8] as described earlier by Abotsi *et al* (2012) [4]. Two sets of experiments were performed to assess the anti-inflammatory activity of the extracts. The first was to study the effects of the drugs given pre-emptively (30 min for i.p. route and 1 h for oral route) before the carrageenan challenge The second examined the effects of the drugs administered 1 h post carrageenan injection. Groups of chicks (n=6) were treated with the extracts suspended in 2 % tragacanth (10-100 mg kg^{-1}, *p.o.*). Diclofenac (10-100 mg kg^{-1}, *p.o.*) was used as a standard Drug vehicle (2 % tragacanth, 10 ml kg^{-1}, *p.o.*) served as a control.

Statistical Analysis

All data are presented as mean± S.E.M (n=6). The time-course curves were subjected to two-way (treatment × time) repeated measures analysis of variance (ANOVA) with Holm-Sidak's post hoc test. Total nociceptive score/total oedema 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.

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

Differences in AUCs were analyzed using one-way ANOVA with drug treatment as a between- subjects factor. Further comparisons between vehicle- and drug-treated groups were performed using the Holm-Sidak's test.

+GraphPad Prism for Windows, Version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P < 0.05$ was considered statistically significant in all analysis.

Results

Phytochemical screening

Preliminary phytochemical screening revealed that all three fractions contained alkaloids, glycosides, sterols and terpenoids. Additionally, HAE had tannins, saponins and flavonoids while EAE contained some tannins.

Acetic acid-induced writhing test

Figure 1 shows the effect of the extracts, morphine and diclofenac on acetic acid-induced writhing during the 25-min observation period. PEE (10-100 mg kg^{-1}, *p.o.*) dose-dependently and significantly ($F_{3,16}$= 3.87, P=0.0312) reduced the number of abdominal writhes over 25 min with a maximum inhibition of 47.44± 9.94 % at 100 mg kg^{-1} (Fig. 1b). EAE (10-100 mg kg^{-1}, *p.o.*) caused a significant ($F_{3,16}$= 5.93; P= 0.0064), dose-dependent reduction in the number of writhes with a maximum inhibition of 66.42±19.12 % at 100 mg kg^{-1} (Fig. 1d). HLE (10-100 mg kg^{-1}, *p.o.*) also significantly ($F_{3,16}$= 7.59; P=0.0016) reduced the number of writhes with a maximum inhibition of 72.43±8.77 % at 30 mg kg^{-1} (Fig. 1f). Additionally, diclofenac, a non-steroidal anti-inflammatory drug (NSAID) (10-100 mg kg^{-1}, *p.o.*) significantly inhibited ($F_{3,16}$= 3.17, P=0.0480) abdominal writhing by a maximum of 44.41±6.72 % (Fig. 2b). Morphine (1-10 mg kg^{-1}, i.p.), an opioid analgesic, profoundly attenuated ($F_{3,16}$= 17.48, P<0.0001) writhing with a maximum inhibition of 100 % at 10 mg kg^{-1} (Fig. 2d). ED_{50} values obtained were 76.05± 27.69 mg kg^{-1} (PEE), 32.6±17.4 mg kg^{-1} (HAE), 14.36±14.83 mg kg^{-1} (EAE), 28.14±95.53 mg kg^{-1} (diclofenac) and 5.00±18.64 mg kg^{-1} (morphine).

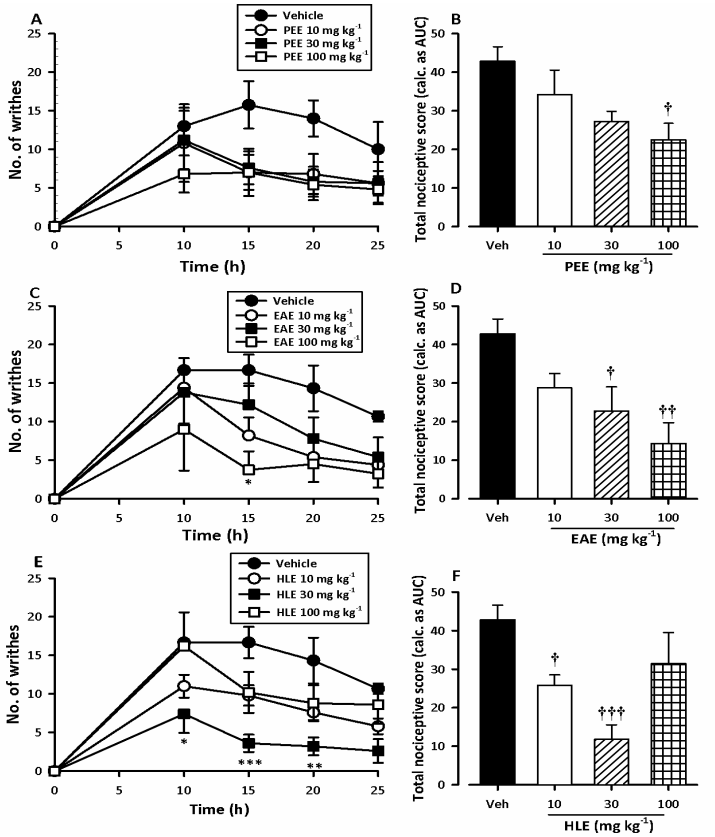

Figure 1: Effect of PEE (10-100 mg kg-1, p.o.), EAE (10-100 mg kg-1 p.o.) and HAE (10-100 mg kg-1 p.o.) on the time course curves (a, c, e) and the total nociceptive score (calc. as AUCs) (b, d, f) of acetic acid-induced writhing in mice. The number of abdominal writhes is shown in 5 min time blocks up to 25 min for the time course curves. Data are presented as mean ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 compared to control group (Vehicle) (Two-way repeated measures ANOVA followed by Holm-Sidak's post hoc test). †P<0.05, ††P<0.01, †††P< 0.001 compared to control group (Veh) (one-way ANOVA followed by Holm-Sidak's post hoc test).

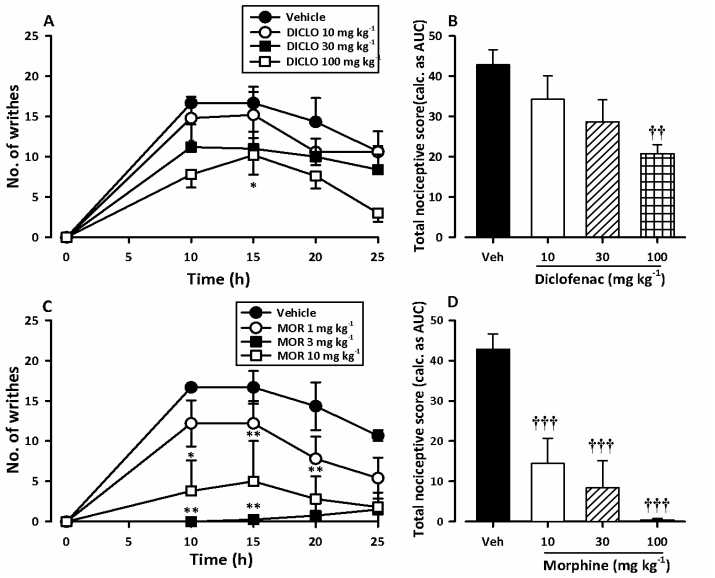

Figure 2: Effect of diclofenac (10-100 mg kg-1 p.o.) and morphine (1-10 mg kg-1, i.p.) on the time course curves (a, c) and the total nociceptive score (calc. as AUCs) (b, d) of acetic acid-induced writhing in mice. The number of abdominal writhes are shown in 5 min time blocks up to 25 min for the time course curves. Data are presented as mean ± SEM (n=6). *P<0.05, **P<0.01 compared to control group (Vehicle) (Two-way repeated measures ANOVA

followed by Holm-Sidak's post hoc test). ††P<0.01, †††P< 0.001 compared to control group (Veh) (one-way ANOVA followed by Holm-Sidak's post hoc test).

Formalin test

Figure 3 shows the effect of the extracts and morphine on formalin-induced pain in mice. Oral administration of HAE (30 mg kg^{-1}), EAE (100 mg kg^{-1}) and PEE (100 mg kg^{-1}) 1h before formalin injection significantly inhibited both the neurogenic ($F_{4,20}$= 6.80, P=0.0014; Fig. 3b) and inflammatory ($F_{4,20}$= 36.23; P<0.0001; Fig. 3c) phases in the formalin test. Morphine (3 mg kg^{-1}, i.p) also significantly inhibited both the neurogenic ($F_{4,20}$= 6.80, P=0.0014; Fig. 3b) and inflammatory ($F_{4,20}$= 36.23; P<0.0001; Fig. 3c) phases in the formalin test.

Theophylline (10 mg kg^{-1}, i.p), completely reversed the anti-nociception of HAE and PEE in both phases of the formalin test (Fig. 4). It, however, did not significantly affect the anti-nociception caused by EAE (Fig. 4) in the formalin test.

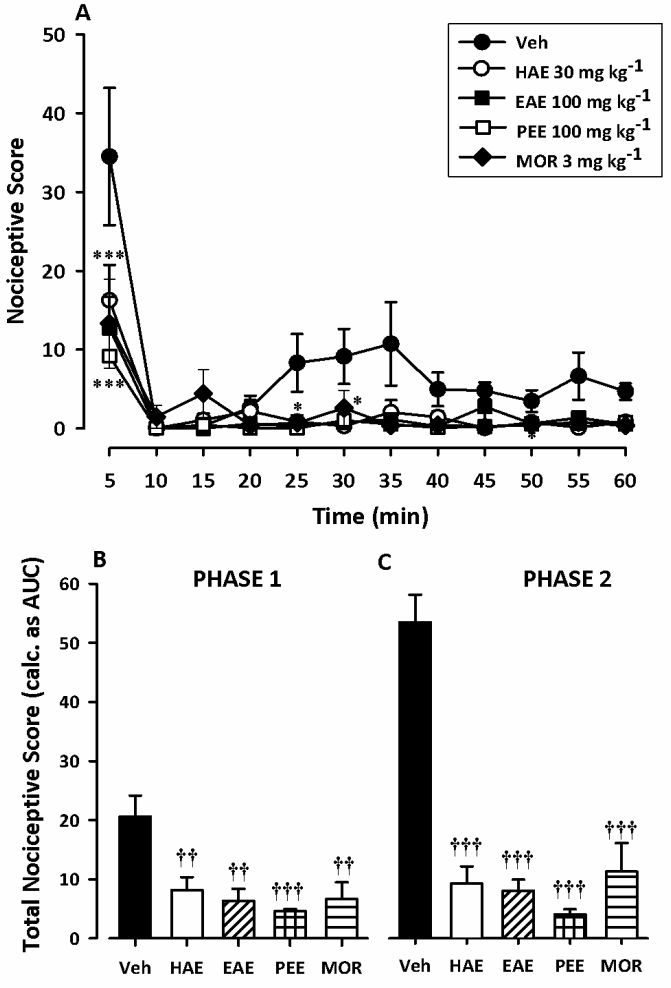

Figure 3: Dose-response effects of PEE (100 mg kg-1, p.o.), EAE (100 mg kg-1 p.o.) and HAE (30 mg kg-1 p.o.) and morphine (3 mg kg-1, i.p.) on formalin-induced licking behaviour in mice. Top panel (a) shows the time course of effects over the 60 min period and the panels below (b, c) show the total nociceptive score calculated from AUCs over the first (0–10 min) and second (10–60 min) phases. Nociceptive scores are shown in 5 min time blocks up to 60 min post-formalin injection. Data are mean±S.E.M (n = 6). *P<0.05, **P<0.01, ***P<0.001 compared to control group (Vehicle) (Two-way repeated measures ANOVA followed by Holm-Sidak's post hoc test). ††P<0.01, †††P< 0.001 compared to control group (Veh) (one-way ANOVA followed by Holm-Sidak's post hoc test).

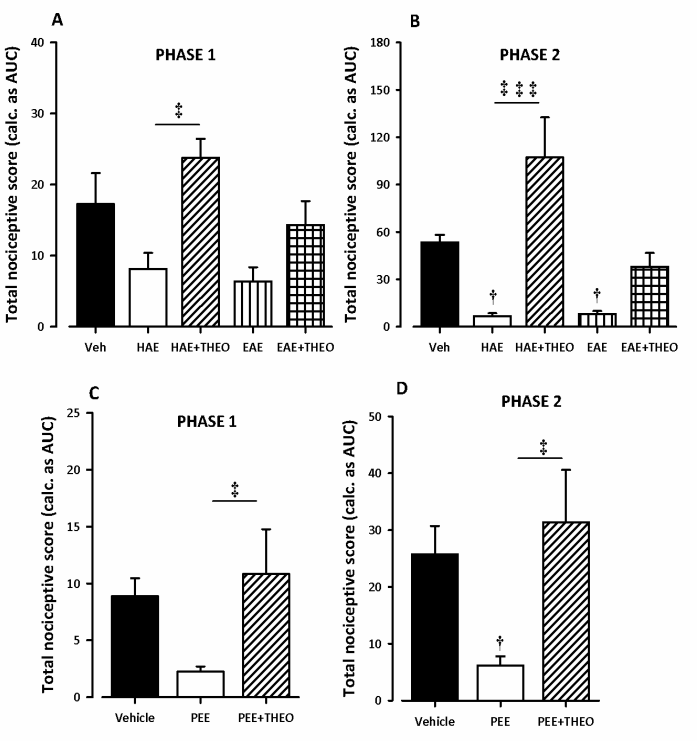

Figure 4: Effect of pre-treatment of mice with theophylline (10 mg kg-1, i.p) on the anti-nociceptive effects of (a, b) HAE (30 mg kg-1, p.o.), (a, b) EAE (100 mg kg-1, p.o.) or (c, d) PEE (100 mg kg-1, p.o.) in the formalin test. Each column represents mean±SEM. (n=6). †P<0.05 compared to respective controls (treated with vehicle); ‡P<0.05; ‡‡‡P<0.001 compared to HAE 30 mg kg-1 or PEE 100 mg kg-1 (one-way ANOVA followed by Holm-Sidak's post hoc test).

Carrageenan-induced Oedema in Chicks

Administration of carrageenan (10 µl, 2 % suspension) induced moderate inflammation resulting in foot oedema in the 7-day old chicks peaking at 2-3 h [8](fig. 5-7). Figures 5 (a, c, e), 6 (a, c, e) and 7 (a, c) show the time course curves for effects of PEE, EAE, HLE and diclofenac respectively on carrageenan-induced oedema. Total oedema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves. HLE (10-100 mg kg^{-1}, *p.o.*) significantly (pre-emptive: $F_{3,\ 17}$= 5.44, P=0.0083; curative: $F_{3,\ 17}$=11.66, P=0.0002) reduced foot oedema with maximal inhibition of 40.96±7.28 % and 64.01±9.21 % for pre-emptive (fig.5f) and curative (fig. 6f) treatments respectively. Similarly, the NSAID diclofenac (10-100 mg kg^{-1}, *p.o.*) significantly (pre-emptive: $F_{3,\ 17}$= 7.65, P=0.0019; curative: $F_{3,\ 17}$=8.17, P=0.0011) and dose-dependently reduced the oedema by a maximum of 48.52±7.02 % and 52.04±5.48 % respectively for pre-emptive (fig.7b) and curative treatments (fig.7d). PEE (10-100 mg kg^{-1}, *p.o.*) showed significant (pre-emptive: $F_{3,\ 19}$= 0.55, P=0.6553; curative: $F_{3,\ 19}$=3.79, P=0.0314) inhibition of oedema only with curative treatment (fig.6b). EAE (10-100 mg kg^{-1}, *p.o.*), did not significantly (pre-emptive: $F_{3,\ 19}$= 1.72, P=0.1959; curative: $F_{3,\ 19}$=2.42, P=0.1001) inhibit foot oedema in the experiments (fig. 5d & 6d).

ED_{50} values obtained were: HLE (pre-emptive: 80.08±30.41 mg kg^{-1}; curative: 16.49±5.31 mg kg^{-1}); diclofenac (pre-emptive: 48.72±49.03 mg kg^{-1}, curative: 10.98±30.41 mg kg^{-1}) and PEE (curative: 44.08±22.53 mg kg^{-1}).

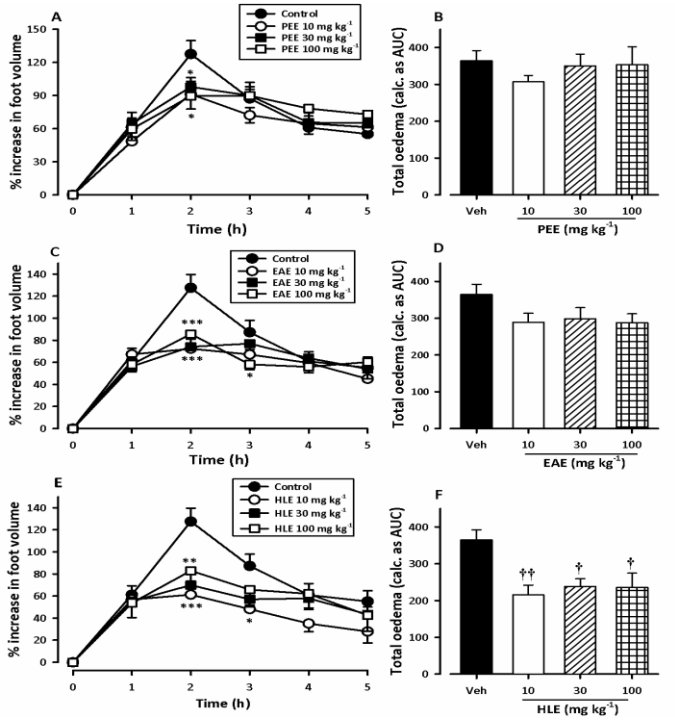

Figure 5: Dose-response effects of PEE (10-100 mg kg-1, p.o.), EAE (10-100 mg kg-1 p.o.) and HAE (10-100 mg kg-1 p.o.) in the pre-emptive protocol of carrageenan-induced foot oedema in chicks. Left panels show the time course of effects and the right panels show the total oedema calculated as AUCs over the 5 h period. Data are means ± S.E.M (n = 6). *P<0.05, **P<0.01, ***P<0.001 compared to control group (Vehicle) (Two-way repeated measures ANOVA followed by Holm-Sidak's post hoc test). †P<0.05, ††P<0.01 compared to control group (Veh) (one-way ANOVA followed by Holm-Sidak's post hoc test).

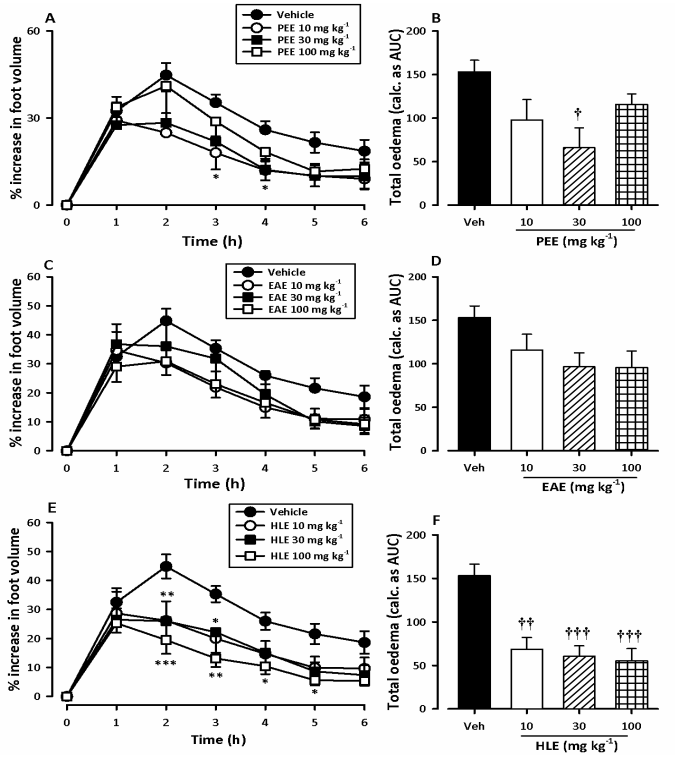

Figure 6: Dose-response effects of PEE (10-100 mg kg-1, p.o.), EAE (10-100 mg kg-1 p.o.) and HAE (10-100 mg kg-1 p.o.) in the curative protocol of carrageenan-induced foot oedema in chicks. Left panels show the time course of effects and the right panels show the total oedema calculated as AUCs over the 6 h period. Data are means ± S.E.M (n = 6). *P<0.05, **P<0.01, ***P<0.001 compared to control group (Vehicle) (Two-way repeated measures ANOVA followed by Holm-Sidak's post hoc test). †P<0.05, ††P<0.01 compared to control group (Veh) (one-way ANOVA followed by Holm-Sidak's post hoc test).

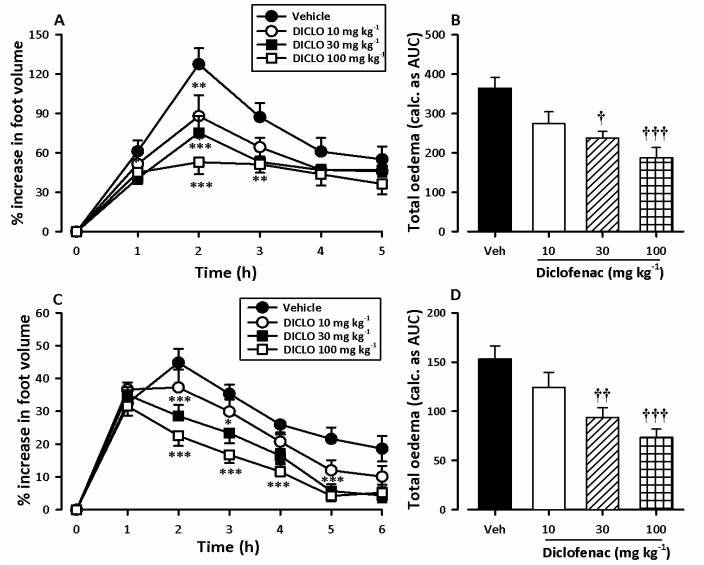

Figure 7: Dose-response effects of diclofenac (10-100 mg kg-1, p.o.) in the pre-emptive (a, b) and curative (c, d) protocols of carrageenan-induced foot oedema in chicks. Left panels show the time course of effects and the right panels show the total oedema calculated as AUCs over the 5-6 h period. Data are means ± S.E.M (n = 6). *P<0.05, **P<0.01, ***P<0.001 compared to control group (Vehicle) (Two-way repeated measures ANOVA followed by Holm-Sidak's post hoc test). †P<0.05, ††P<0.01, †††P<0.001 compared to control group (Veh) (one-way ANOVA followed by Holm-Sidak's post hoc test).

Discussion

The present study is a follow-up on our earlier work [3, 4] in which we showed that the hydro-ethanol extract of the aerial parts of *Hilleria latifolia* has anti-nociceptive and anti-inflammatory effects in animals. In this study, we have investigated the anti-nociceptive and anti-inflammatory effects of three extracts obtained from the aerial parts of *Hilleria latifolia* with the aim of eventually identifying the active constituents in the plant.

The anti-nociceptive effects of the various extracts were tested in the acetic acid abdominal writhing and formalin tests. The acetic acid abdominal writhing test was used first since it shows good sensitivity and allows for the detection of the effects of weak analgesics.[9, 10] All three extracts exhibited significant analgesic properties on oral administration. Intraperitoneal injections of acetic acid induces nociception though activation of chemosensitive nociceptors, or visceral surface irritation, leading to release of inflammatory mediators, including histamine, bradykinin, prostaglandin (PGE_2 and $PGF_{2\alpha}$), serotonin and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8.[11-14] The analgesic activity of the extracts may be attributed to attenuation of the release or actions of the pro-inflammatory mediators.

The analgesic properties of the extracts were confirmed in the formalin test. The formalin test is one of the most predictive of acute pain and a valid model of clinical pain.[9, 15] The formalin test produces a distinct biphasic nociceptive response. An early phase (neurogenic pain), occurring within seconds of formalin

injection, is elicited by direct chemical activation of nociceptive primary afferent fibres. A second, later phase (inflammatory pain), occurs as a result of ongoing activity in primary afferents, the release of inflammatory mediators and a glutamate-dependent sensitization of nociceptive spinal neurones.[16, 17] It has been suggested that centrally acting drugs such as opioids inhibit both early and late phases almost equally; most NSAIDs and corticosteroids, which are primarily peripherally acting, only inhibit the late phase.[15] The attenuation of nociception in both phases suggests that the extracts have central and peripheral actions. Theophylline significantly antagonised the anti-nociceptive effects of HAE and PEE suggesting a possible adenosinergic pathway involvement in the actions of HAE and PEE. According to literature,[18, 19] the activation of A_1 adenosine receptors, peripherally, spinally and supraspinally, produces anti-nociception. Since theophylline blocks adenosine A_1 and A_2 receptors, the anti-nociceptive effects of HAE and PEE may involve activation of A_1 receptors and/or an increment in endogenous adenosine either centrally or peripherally.

The anti-inflammatory activities of the extracts were assessed with the carrageenan-induced oedema test in chicks.[4, 8] The inflammatory response induced by carrageenan is characterized by a biphasic response [20] with marked oedema formation resulting from the rapid production of several inflammatory mediators such as histamine, serotonin and bradykinin (first-phase), which is subsequently sustained by the release of prostaglandins and nitric oxide (second-phase) with peak at 3 h, produced by inducible isoforms of COX (COX-2) and nitric oxide synthase (iNOS), respectively.[21, 22] The second (late) phase is sensitive to most clinically effective anti-inflammatory drugs.[20, 23] From the study, it was only HAE that significantly inhibited inflammation on both prophylactic and curative oral treatment. PEE showed significant anti-inflammatory activity only on curative but not prophylactic treatment. HAE appeared to have more activity in the late phase of the oedema than the early phase—suggesting possible action against nitric oxide, prostaglandins and other cyclooxygenase products. The exact mechanism, however, needs to be established.

The anti-nociceptive and anti-inflammatory activities shown by the various extracts in this study suggest the role of multiple active constituents with analgesic and anti-inflammatory properties. In general, HAE appeared to be the most effective in attenuating pain and inflammation in the models used. Efforts are ongoing in our laboratory to further fractionate HAE so as to identify the active compounds in it.

Conclusion

In conclusion, the hydroalcoholic, ethyl acetate and petroleum ether extracts of the aerial parts of *Hilleria latifolia* have anti-nociceptive properties in rodents. The hydroalcoholic and petroleum ether extracts also possess anti-inflammatory activity. Also, the mechanisms of anti-nociceptive action of the hydroalcoholic and petroleum ether extracts involve an interaction with the adenosinergic pathway. The hydroalcoholic extract was generally the most efficacious of the three extracts.

Conflict of Interest

None declared.

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