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Anti-inflammatory, anti-nociceptive and antipyretic activity of young and old leaves of *Vernonia amygdalina*

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ABSTRACT

Background: Both young and old leaves of *Vernonia amygdalina* (VA) are traditionally used to treat inflammation, pain and fever. However, the efficacy of young and old leaves for treating these ailments have not been compared till date.

Aim: To ascertain the effect of young and old leaves of VA in managing inflammation, pain and fever.

Methods: Both quantitative and qualitative phytochemical screening of ethanol extracts of young (EthYL) and old (EthOL) leaves of VA were performed. The anti-inflammatory activity of orally administered EthYL and EthOL (50–200 mg/kg) and Diclofenac (10 mg/kg) were evaluated in carrageenan-induced inflammation model in rats. Antipyretic activity of EthYL, EthOL and Aspirin (25 mg/kg) were assessed in the Baker's yeast-induced pyrexia model. Anti-allodynic effect of both extracts were evaluated by inserting inflamed paws of rats in cold water. Antinociceptive property of the extracts were assessed using tail withdrawal and formalin-induced nociception test. Histopathological examination of the paws was performed, in addition to formalin test to understand the possible mechanism of action of the extracts. Negative control rats received 2 ml/kg normal saline in all tests.

Results: The amount of flavonoids, alkaloids, tannins, and phenolics were significantly ($p < 0.05$) higher in EthOL than EthYL, while saponins were significantly higher ($p < 0.05$) in EthYL than EthOL. The antioxidant ability and total antioxidant capacity were significantly ($p < 0.05$) higher in EthYL than EthOL. However, this was significantly ($p < 0.05$) lower than the anti-oxidant activity of Ascorbic acid. A dose-dependent increase in anti-inflammatory, antipyretic and antinociceptive properties were observed in both EthYL and EthOL, similar to the standard drugs. Mast cell degranulation accompanied by vasodilatation and high leukocytosis were observed in the negative control, but were markedly low in extract treated groups. Both extracts mediated their analgesic effect through opioidergic and nitric oxide pathways with EthYL additionally implicating the muscarinic cholinergic system.

Conclusion: Although both EthYL and EthOL alleviate inflammation, pyrexia and nociception, EthYL of VA was found to be more potent than EthOL.

1. Introduction

Inflammation is an immunological response to infection, damaged cells, irritants, mechanical and chemical injuries, in an attempt to eliminate injurious stimuli and initiate a healing process of the damaged tissue [1,2]. Pain, swelling (edema), heat and redness are the four cardinal signs associated with acute inflammation [3,4]. An increase in vascular permeability, as seen in acute inflammation, results in the exudation of plasma rich fluid, accompanied by the inflow of proteins such as albumin into the damaged tissue area. The result is an

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edematous lesion [3,5]. Pain is associated with many ailments and is usually the main factor that prompts patients to seek medical attention [6]. Pain occurs as a result of nociceptive, inflammatory or neuropathic conditions [7], while pyrexia or fever manifests as a secondary impact of infection or tissue damage as seen in inflammation, graft rejection, tumour malignancy or other diseased states [5,8]. The infected or damaged tissue initiates the enhanced synthesis of pro-inflammatory mediators or cytokines such as interleukin -1β and tumor necrosis factor (TNF)-α, which increases the production of prostaglandin E2 (PGE2) near the area of preoptic hypothalamus. PGE2 then sends signals to the hypothalamus which elevates the body temperature beyond the set point of 37.5–38.3 °C in the anus or rectum, 37.7 °C in the mouth and 37.2 °C under the arm or axillar [5,9,10]. Most antipyretic agents exert their temperature-reducing effect by inhibiting PGE2 biosynthesis [11].

Although there are many proven and effective drugs on the market for the management of inflammatory conditions, they are accompanied with varying toxic and life-threatening side effects [12,13]. It is, therefore, prudent that much more viable treatments with milder toxic effects, reliable, efficacious and cost-effective herbal-based products made available to the populace due to their continuing wide usage; be investigated, and possibly used as alternative means of such management.

The leaves of *Vernonia amygdalina* (VA) have found relevance in traditional folk medicine for many years as antipyretic, antimalarial, antimicrobial, antidiabetic, anthelminthic, an immune system booster, energizer, and as a pain reliever [14–18]. In recent years, interest in the constituents of VA as potent anti-inflammatory and antimalarial agents have attracted both research [19–21] and clinical [22] attention. Whereas some therapeutic abilities of the mixture of young and old leaves of VA have been given scientific credence [19,21], there is no literature backing the anti-inflammatory efficacy of these leaves when used singly or separately. We have previously demonstrated that the young leaves of VA possess higher antioxidant and antidiabetic potentials as compared to the old leaves [15].

This study thus seeks to ascertain the pharmacotherapeutic usefulness of young and old leaves of VA in the management of pain, fever and edema caused by acute inflammation, and also report for the first time, the quantitative analysis of the young and old leaves of VA.

2. Materials and method

2.1. Drugs and chemicals

Carrageenan and 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical (Sigma-Aldrich Inc., St. Louis, MO, USA); baker's yeast (Saf-instant, S.I. Lesaffre, France); Diclofenac sodium and Aspirin (Troge Medical GmbH, Hamburg, Germany); formalin, N^G-L-nitro-arginine methyl ester (L-NAME) (Sigma-Aldrich Inc., St. Louis, MO, USA); glibenclamide (Daonil®, Sanofi-Aventis, Guildford, UK); yohimbine hydrochloride (Procomil®, Walter Ritter GmbH + Co. KG, Germany); atropine sulphate, naloxone hydrochloride, morphine sulphate (Duopharma (M) Sdn Bhd, Malasia) and nifedipine (Denk Pharma, Germany). All drugs and reagents were of high standard grade and freshly prepared before used.

2.2. Collection and preparation of ethanolic leaf extracts

V. amygdalina leaves (bitter leaf) were collected from the Botanical Garden of the School of Biological Sciences, University of Cape Coast (UCC), Cape Coast, Central Region of Ghana, and were subsequently identified by a botanist at UCC. Samples were deposited in the School's herbarium, with the accession number CE 001. The leaves were harvested from the same plants and then grouped into young leaves (YL) and old leaves (OL) following the criteria described previously [15,23]. Furthermore, the average number of days by which the YL develops into OL was evaluated. Here, the lower limit by length of YL (5.10 cm) was chosen, and petioles of young vegetative leaves with this length were identified and marked with an indelible marker. These YL of VA were measured every day until they got exactly to or just above the upper limit by length (15.50 cm) of the old vegetative leaves. The average number of days was estimated to be 21 days (range: 19–23 days).

For the extract preparation, briefly, VA leaves (both YL and OL) were air-dried separately for two weeks, pulverized and stored in air-tight plastic containers. Powdered YL and OL were macerated in cold ethanol for 72 h, separately, and the extracts were collected. This process was repeated thrice for exhaustive extraction and the extracts were concentrated to dryness using a rotary evaporator. The extract concentrates were labelled and percentage yield was calculated in %w/w and stored at 4 °C in sealed containers until further use.

The yield was 19% w/w for ethanolic leaf extract of YL (EthYL) and 22% w/w for ethanolic leaf extract of OL (EthOL). Concentrations (50, 100, and 200 mg/ml) of both extracts were prepared from the stored extracts for oral administration.

2.3. Phytochemical screening

The chemical classes of the bioactive compounds present in EthYL and EthOL were investigated using standard phytochemical screening procedures, according to the methods described previously [24,25]. Quantitative analysis of the phytochemicals screened was done using standard procedures explained earlier [26–30].

2.4. In vitro antioxidant activity

The antioxidant activity of EthYL and EthOL extracts was assessed by DPPH free radical assay [31]. The reaction mixture containing 1 ml of varying concentration of EthYL or EthOL was mixed with 3 ml of methanol and 0.5 ml of 1 mM DPPH. The mixture was shaken vigorously and left to stand in the dark. The decrease in the DPPH-radical was measured at 517 nm by UV–vis spectrophotometer (Evolution™ 60S, Thermo Scientific, Germany). Ascorbic acid (vitamin C) was used as a standard antioxidant. Concentrations of 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 mg/ml EthYL and EthOL were prepared in methanol. Ascorbic acid (vitamin C) was used as a standard antioxidant at the concentrations 0.05, 0.1, 0.2, 0.5, and 0.75 mg/ml. The antioxidant activity of EthYL and EthOL was calculated using the formula:

$$\%\, inhibition = \frac{Ab - Aa}{Ab} \times 100$$

where 'Ab' = absorption of the blank sample and 'Aa' is the absorption of the extract.

2.5. Experimental animals

Sprague-Dawley rats weighing 250 ± 50 g and ICR mice weighing 21–38 g, of both sexes, were randomly selected from the animal house of the Department of Biomedical Sciences, University of Cape Coast (UCC). The animals were kept in stainless steel cages (34.5 × 47.6 × 18.5 cm) with soft wood shavings as bedding material, under ambient conditions of temperature (23 ± 2 °C), relative humidity (60–70%), 12 h light/dark cycle and water and rat/mice chow were available *ad libitum*. However, preceding drug administration, animals were fasted overnight but were still allowed free access to clean water.

All animals used were handled in accordance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, 1985, revised 1996). All protocols used were approved by the Departmental Ethics Committee.

2.6. Carrageenan-induced paw edema in rat

Anti-inflammatory evaluation was done using the carrageenan-induced paw edema method as described previously [32]. Edema was induced by injecting 100 µl of freshly prepared 2% carrageenan into the subplantar surface of the left hind paw of 8 groups rats (n = 5) whereas the sham control (9th) group rats received intraplantar injection of vehicle (100 µl of normal saline). Prior to the induction of inflammation, baseline recordings were taken by measuring the diameter of the non-inflamed left hind paw of the rats, using a digital caliper. The diameter of the inflamed left hind foot was measured one hour later to assess the effect of carrageenan on the size of the foot. The rats in the various groups were then dosed with the extracts, EthOL and EthYL, at 50, 100, and 200 mg/kg, *p.o,* diclofenac (10 mg/kg, *p.o.*) or normal saline (10 ml/kg, *p.o.*). One hour after treatments, the diameter of the left hind paw of the rats were measured at every one hour for 4 h, to ascertain the effect of VA on the inflamed left paw. Raw scores of the foot edema were individually normalized as percentage of change in their paw diameter at time 0 and then averaged for each treatment group as follows:

$$\%\ change\ in\ paw\ edema = \frac{Paw\ thickness\ at\ time\ t - Paw\ thickness\ at\ time\ 0}{Paw\ thickness\ at\ time\ 0} \times 100$$

This was used to plot a time course curve for the 5 - h period. Total edema response for each treatment was then calculated as area under the time course curves. The effect of the drugs was evaluated using percentage inhibition of edema calculated using the formula below:

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

2.7. Carrageenan-induced cold allodynia

The test was carried out as described earlier [33]. The temperature of cold water was set at 4 °C ± 0.5 °C and allowed to stabilize for 5 min. Each hind paw of the animals was then placed into the cold water and the time (in seconds) taken for the first brisk lift paw to occur was recorded (baseline). The time to the brisk response was interpreted as the latency for cold pain withdrawal.

A maximum cut-off time of 10 s was used to prevent tissue damage at the lower temperatures. An amount of 100 µl of 2% carrageenan was then injected into the left hind paw of the rats to induce inflammation. 2 h post carrageenan injection, the rats were put into eight groups (n = 5), and were treated orally as follows: Group A (positive control, Diclofenac, 10 mg/kg), Group B, (the negative control, normal saline, 2 ml/kg) Groups C, D and E were treated orally with EthYL at doses of 50, 100, and 200 mg/kg, respectively, whereas Groups F, G and H were received EthOL at doses of 50, 100, and 200 mg/kg. The sham control (SC) group rats received intraplantar injection of vehicle (100 µl of normal saline) and were subsequently treated with normal saline, 2 ml/kg. 1 h after the various drug treatments, the test was carried out again at 1 h intervals for 4 h to ascertain the effect of VA on induced pain. The latency to paw withdrawal of the rats to allodynia was used to calculate the percentage maximum possible effect (MPE) of the extracts, at a cut-off latency of 10 s.

$$\%MPE = \frac{Total\ Treatment\ Response\ Time - Total\ Baseline\ Response\ Time}{Cut - off\ Latency\ Time - Total\ Baseline\ Response\ Time} \times 100$$

This was used to plot a time course curve for the 4 - h period. Total anti-nociceptive effect for each treatment was then calculated as area under the time course curves.

Table 1
Total phenolic content, flavonoids, tannins, total antioxidant capacity, saponins and percentage alkaloids of ethanolic extract of young and old leaf parts of *V. amygdalina*.

Phytochemical	EthYL	EthOL	*p*-value
Total phenolics	0.165 ± 0.002	0.212 ± 0.011	0.0021***
Flavonoids	0.377 ± 0.008	0.663 ± 0.061	< 0.0001****
Tannins	1.555 ± 0.009	1.925 ± 0.187	< 0.000017****
TAC	0.245 ± 0.031	0.150 ± 0.012	< 0.0001****
Saponins	34.480 ± 0.667	29.390 ± 2.514	< 0.00001****
Alkaloids	2.672 ± 0.018	5.332 ± 0.018	< 0.0001****

Data is shown as mean ± SEM; (n = 2). Significance in difference between YL and OL extract of VA. P < 0.05 (unpaired t-test with Welch's correction). TAC: Total antioxidant capacity.

2.8. Tail immersion test

The test was carried out as described earlier [20]. This experiment was done using hot water maintained at 55 ± 0.5 °C in a bath. Briefly, rats were put into eight groups, A–H (n = 5). Group A was treated orally with 10 mg/kg diclofenac, Group B, was treated orally with normal saline (2 ml/kg), Groups C, D and E were treated orally with EthYL extracts at doses of 50, 100, and 200 mg/kg respectively, whereas Groups F, G and H were treated orally with EthOL extracts at doses of 50, 100, and 200 mg/kg. The 5 cm length from the tip of the tail of the rats was immersed into the hot water maintained at 55 ± 0.5 °C with the rats held vertically above the water bath. The cut-off time for the test was set to 10 s after which the subjects were withdrawn to prevent tissue damage. The time (in seconds) from the dipping of tail into the hot water and rapid flick/withdrawal from hot water was recorded. The percentage maximum possible effect (MPE) of the extract was calculated using a similar formula as indicated above under cold allodynia.

2.9. Formalin test

This test was performed as described previously [34,35]. Eight groups (n = 5) of ICR mice were used for the test. Each mouse was placed in one of twenty Perspex chambers (15 × 15 × 15 cm) for one hour prior to formalin injection for them to acclimatize to the new environment. Each group of mice received either vehicle (10 ml/kg distilled water, *p.o.*), EthOL (50, 100 and 200 mg/kg, *p.o.*), EthYL (50, 100 and 200 mg/kg, *p.o.*) or morphine (3 mg/kg, i.p.) 60 min (*p.o.*) or 30 min (i.p.) before intraplantar injection of 5% $^{v}/_{v}$ formalin (10 µl) into their right hind paws. Mice were instantly transferred into the transparent testing chamber. They were then captured with the aid of a camcorder for 60 min A nociceptive score for every 5 min time bloc was obtained by measuring the duration and frequency of licking/biting of injected paws and the mean nociceptive score for each time bloc per 5 min determined as the product of the duration and frequency of licking/biting. The results obtained were considered as early/neurogenic phase (0–10 min) and late/inflammatory phase (10–60 min) from which time-course curves were plotted and the areas under the curve for each phase and each treatment determined and plotted. The percentage inhibitions of the treatment groups were obtained using a similar formula as described under paw edema. The results were also used to plot a dose-response curve from which the ED_{50} were determined. Excised tissues were also taken from the right hind paws from each group for histological analysis.

2.10. Yeast-induced pyrexia model in rats

Yeast-induced pyrexia model [36] was used to assess the antipyretic potential of EthYL and EthOL in Sprague-Dawley rats. Nine groups of rats (n = 5) labeled A–I were used for the study. Group A, the sham

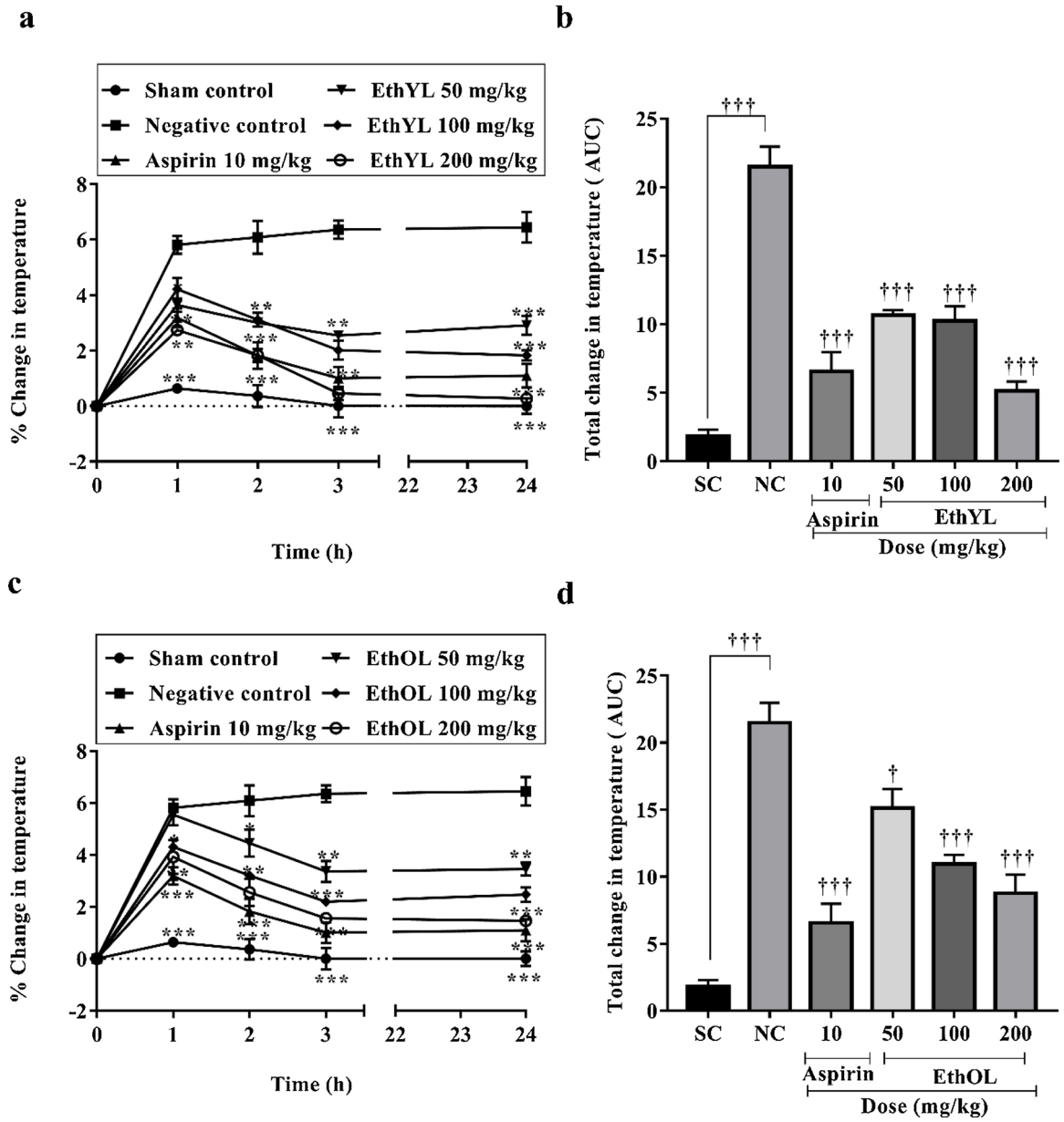

Fig. 1. Effect of EthYL and EthOL (50, 100, and 200 mg/kg) treatments on yeast-induced pyrexia in rats. "a and c", show the percentage change in temperature over time in EthYL and EthOL treated groups, respectively. "b and d" represent the total percentage pyrexia calculated as AUC in yeast-induced pyrexia in rats. Data is plotted as mean ± SEM. *$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$; comparing drug treatments to the negative control (two-way ANOVA followed by Dunnet's *post-hoc* test). †$P \leq 0.05$; ††$P \leq 0.01$; and †††$P \leq 0.001$; comparing drug treatments to the negative control (one-way ANOVA followed by Newman-Keuls *post-hoc* test).

control, were treated orally with 2 ml/kg normal saline. Group B, the negative control was treated orally with 2 ml/kg normal saline whereas Group C, the positive control, was treated with 25 mg/kg aspirin. Groups D, E, and F were treated orally with EthYL at doses of 50, 100, and 200 mg/kg respectively, whereas Groups G, H, and I were treated orally with EthOL at doses of 50, 100, and 200 mg/kg respectively. Before pyrexia was induced, baseline rectal temperatures of the rats were taken using a digital thermometer. Pyrexia was induced by multiple intraperitoneal injections of baker's yeast suspension (0.135 g/kg), after which the animals were fasted for 18 h. Rectal temperatures were then taken at the 18th and 19th hours after which EthYL, EthOL, normal saline and aspirin were administered to rats with a 0.5 °C rise in temperatures. Rectal temperatures were again taken at the first, second and third hours post-treatment, and then the next morning. Raw scores for basal and changes in rectal temperatures were individually normalized as percentage of change from their values at time 0, and then averaged for each treatment group. Time-course curves were then plotted from which the total change in rectal temperature for each treatment was calculated in arbitrary unit as the area under the curve (AUC). The percentage inhibition for each treatment group was calculated as described under Section 2.6.

2.11. Mechanism of action of extracts

The mechanisms mediating the analgesic effect of EthOL and EthYL were assessed using opioidergic, ATP sensitive K^+ channels, nitric oxide, muscarinic, adrenergic and voltage-gated calcium channel pathways in the formalin test as described previously [37,38]. Selection of doses for the test was based on preliminary studies in our lab [39,40].

The involvement of the above pathways was assessed by pre-treating mice (n = 5) with naloxone (a non-selective opioid receptor antagonist, 2 mg/kg, i.p.), atropine, (a non-selective muscarinic

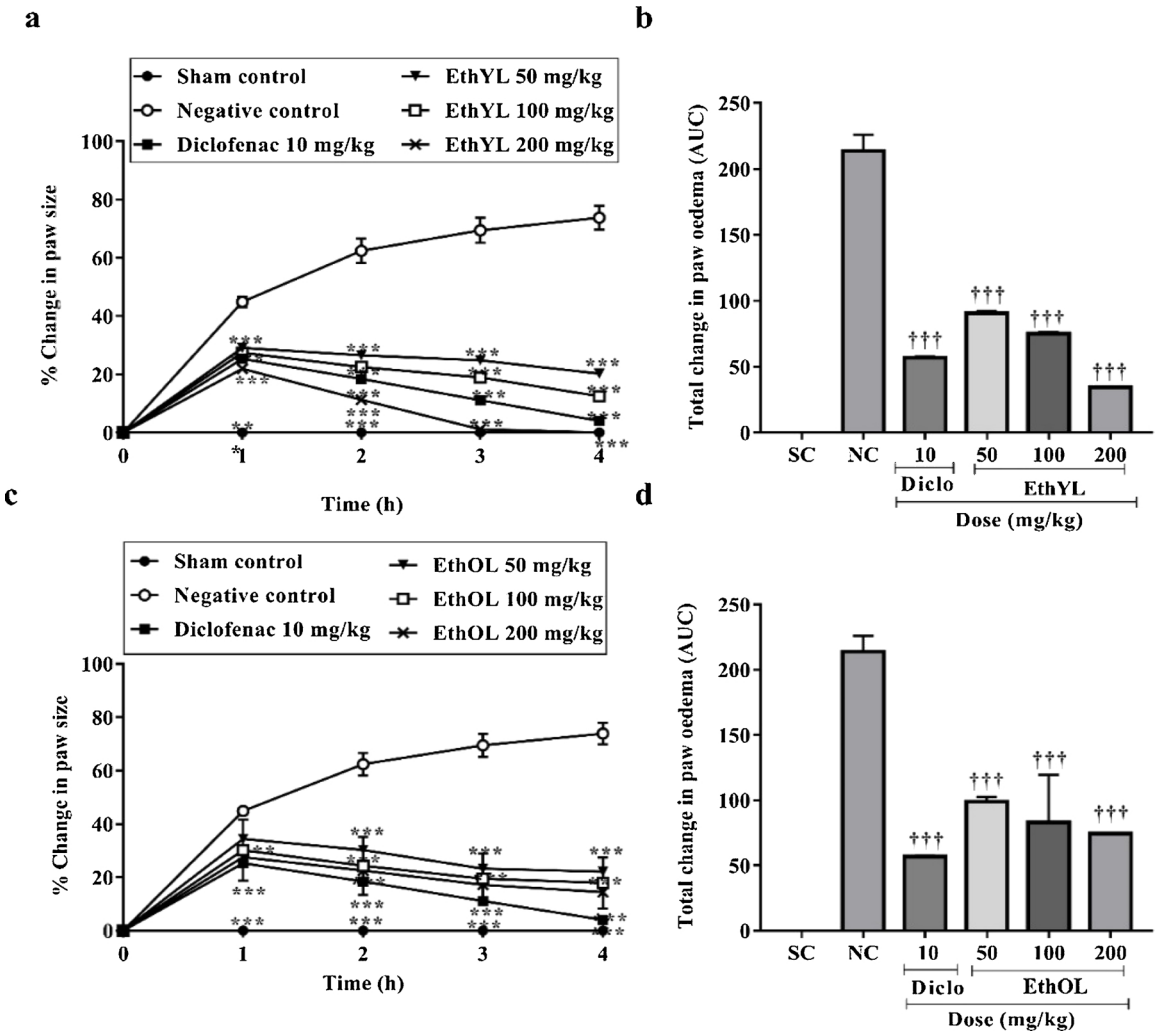

Fig. 2. Effects of EthYL and EthOL (50, 100, and 200 mg/kg) treatments on carrageenan-induced paw edema in rats. "a and c" show the percentage change in edematous paw size in EthYL and EthOL treated groups, respectively, over time. "b and d" represent the total percentage edema calculated as AUC in carrageenan-induced edema. Data is plotted as mean ± SEM. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and $^{***}P \leq 0.001$; comparing drug treatments to the negative control (two-way ANOVA followed by Dunnet's *post-hoc* test). $\dagger P \leq 0.05$; $\dagger\dagger P \leq 0.01$; and $\dagger\dagger\dagger P \leq 0.001$; comparing drug treatments to the negative control (one-way ANOVA followed by Newman-Keuls *post-hoc* test).

antagonist, 5 mg/kg, i.p.), nifedipine (10 mg/kg, *p.o.*, L-type voltage-gated calcium channel blocker), glibenclamide (an ATP-sensitive potassium channel inhibitor, 8 mg/kg, *p.o.*), L-NAME (10 mg/kg, i.p., a nitric oxide synthase inhibitor), yohimbine, (an alpha-2 receptor antagonist, 3 mg/kg, *p.o.*) or vehicle. After 15 min (i.p) or 30 min (*p.o.*), mice received EthOL (200 mg/kg, *p.o.*), EthYL (200 mg/kg, *p.o.*), morphine (3 mg/kg, i.p.) or vehicle (10 mL/kg, *p.o.*). After 1 h (*p.o.*) or 30 min (i.p.), the nociceptive responses to formalin were recorded, with the aid of a camcorder for 60 min., and analysed similar to formalin test results.

2.12. Histological analyses

Mice that received formalin injection subcutaneously into their right hind paw were euthanized after 2 h (after phase 2). Excised tissues from the right hind paws were fixed in 10% phosphate buffered formalin. They were subsequently dehydrated, cleared and embedded in paraffin wax to form tissue blocks. Serial sections were taken using a rotary microtome and stained using routine haematoxylin and eosin (H&E) and toluidine blue. H&E staining were employed for analysing leucocytic cell infiltrates, vasodilation and congestion; while the special stain (Toluidine blue) was used for evaluating mast cell degranulation dynamics.

2.13. Statistical analyses

GraphPad Prism Version 7 was used for data analyses. Data are presented as mean ± standard error of mean (mean ± SEM). Differences in data for sham, treatments, and the negative control (NC) were analyzed using ANOVA. Further, comparisons were performed using Dunnett's *post-hoc and* Newman Keuls' *post hoc* tests. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical screening and antioxidant activity

The results from the phytochemical screening of both EthYL and EthOL is shown in S Table1. Both extracts were positive for alkaloids, tannins, saponins, cardiac glycosides, terpenes and steroids. EthYL extracts were negative for flavonoids, while EthOL were positive for flavonoids. Quantitative analysis revealed the presence of significantly higher ($p < 0.05 - 0.001$) quantities of total phenolics, flavonoids, tannins and alkaloids in EthOL than in EthYL. Phytochemical assay is a

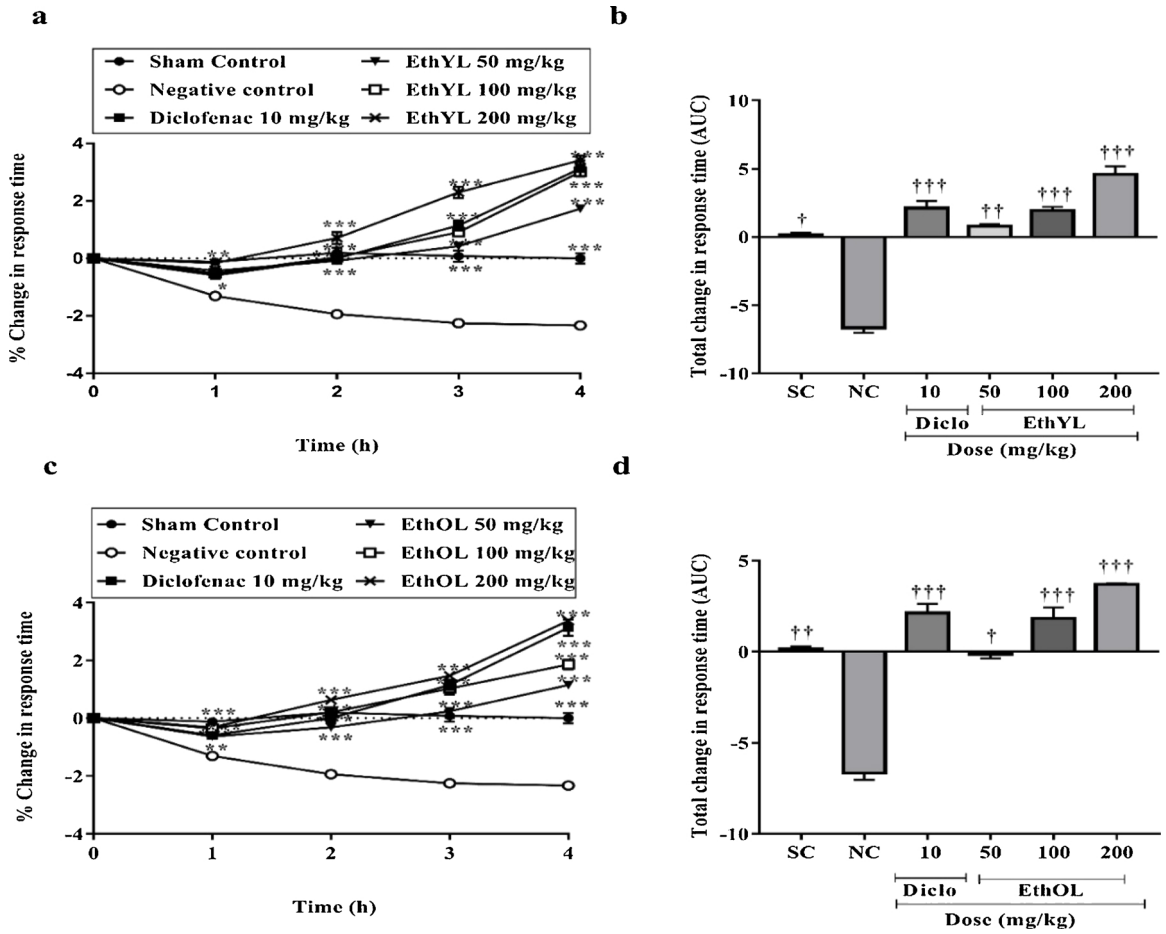

Fig. 3. Effects of EthYL and EthOL (50, 100, and 200 mg/kg) treatments on carrageenan induced cold allodynia. "a and c" represent the percentage change in response time to cold allodynia and "b and d" the total change in response time to cold allodynia calculated as AUC.
*$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$; comparing drug treatments to the negative control (two-way ANOVA followed by Dunnet's *post-hoc* test). †$P \leq 0.05$; ††$P \leq 0.01$; and †††$P \leq 0.001$; comparing drug treatments to the negative control (one-way ANOVA followed by Newman-Keuls *post-hoc* test).

less sensitive assay and the presence of low quantities of flavonoids might have been insufficient to trigger the chemical reaction. The total antioxidant capacity (TAC) and saponins were significantly higher in EthYL than in EthOL ($p < 0.01$) (Table 1).

3.2. Effect of EthYL and EthOL on yeast-induced pyrexia

Pyrexia was observed 21 h post-induction with yeast in the rats (Sprague-Dawley), with a mean rectal temperature of 36.57 ± 0.17 °C which increased to 38.17 ± 0.63 °C. Treatment with 200 mg/kg EthYL and EthOL extracts induced a significant ($p < 0.05$) inhibition of pyrexia by 78.54% and 31.78%, respectively; while Aspirin, the reference drug, inhibited pyrexia by 71.26%. No significant change in rectal temperature was observed in the Normal control group (Fig. 1). A dose-dependent antipyretic activity was exhibited by both EthYL and EthOL extracts. However, EthYL extracts were found to have better antipyretic activity than EthOL.

3.3. Effect of EthYL and EthOL on edema

Edema was observed two hours post-induction by carrageenan (2%) in the left hind paw. A mean of 73.83% increase in paw size was observed in the negative control group as shown on Fig. 2a and c. Treatment with Diclofenac (10 mg/kg; reference anti-inflammatory drug) reduced the total edematous paw size significantly by 76.52% ($p < 0.01$). Treatment with EthYL extracts (50, 100, and 200 mg/kg) significantly ($p < 0.05$) exhibited a dose-dependent reduction in edema (59.61%, 67.52% and 86.31%) 2 h post-treatment. Similarly, treatment with EthOL (50, 100 and 200 mg/kg) also exhibited a significant ($p < 0.05$) dose-dependent reduction in edema (56.11, 63.37% and 67.41%, respectively) 2 h post-treatment (Fig. 2c and d).

3.4. Effect of EthYL and EthOL on pain

3.4.1. Carrageenan induced cold allodynia

A dose-dependent decrease in cold allodynia was observed with both EthYL and EthOL extracts, after 2 h of treatment. Both 100 and 200 mg/kg EthYL and EthOL extracts significantly ($P \leq 0.05$) inhibited the carrageenan induced cold allodynia in the rats, similar to Diclofenac ($P < 0.05$), the reference analgesic (Fig. 3 and S Table 2). Again, the EthYL extracts showed better inhibition of the cold allodynia than the EthOL extracts.

3.4.2. Tail immersion test

The analgesic activity of EthYL and EthOL measured by tail-immersion test is shown in Table 3. Treatment with both EthYL (50, 100 and 200 mg/kg) and EthOL (200 mg/kg) increased the tail withdrawal latency significantly ($P \leq 0.05$) (Fig. 4 and S Table 3). Diclofenac was used as a standard for this test.

3.4.3. Formalin test

Pain reaction in formalin induced pain model is usually observed as a biphasic reaction – first phase (neurogenic phase) characterized by an immediate, intense pain response (0–10 min) and the second phase

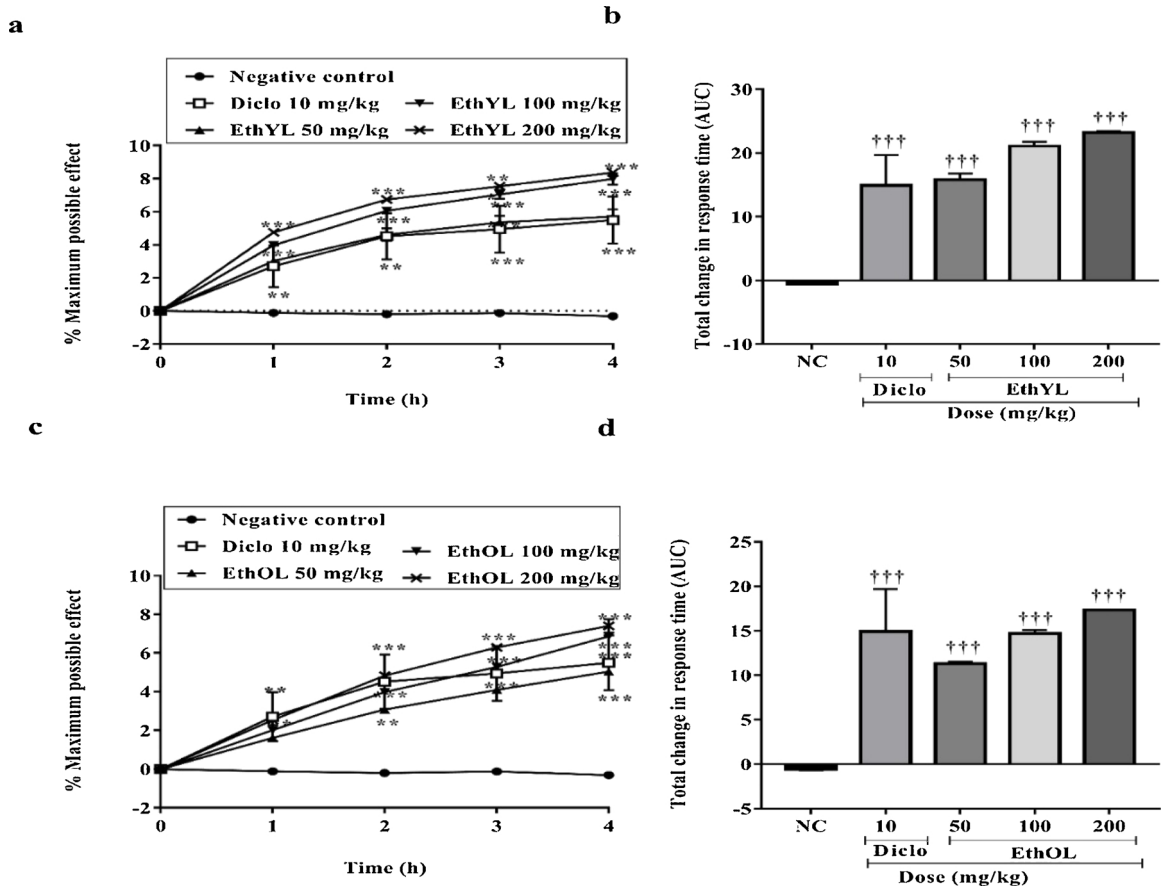

Fig. 4. Effect of EthYL (50, 100, and 200 mg/kg) and EthOL (50, 100, and 200 mg/kg) treatments, on time course curves of latency to tail withdrawal from hot water (a and c) and (b and d) the total anti-nociceptive score calculated as the AUC. Values plotted are mean ± SEM. *$P \leq 0.05$; **$P \leq 0.01$; and ***$P \leq 0.001$; comparing drug treatments to the negative control (two-way ANOVA followed by Dunnet's *post-hoc* test). †$P \leq 0.05$; ††$P \leq 0.01$; and †††$P \leq 0.001$; comparing drug treatments to the negative control (one-way ANOVA followed by Newman-Keuls *post-hoc* test).

(inflammatory) is characterized by delayed pain due to the release of inflammatory mediators (10–60 min). A dose-dependent decrease in the licking time was observed in morphine, EthYL and EthOL treated groups. Oral administration of both EthOL and EthYL one hour prior to formalin injection produced a significant antinociceptive effect (all $P < 0.05$). At 200 mg/kg, both EthOL and EthYL produced a maximum inhibition of 65.1 ± 3.65% and 76.97 ± 3.25%, respectively, in the phase I; and 77.91 ± 3.95% and 92.57 ± 0.86% in the inflammatory phase. Morphine (3 mg/kg, i.p.) was used as a reference drug and significantly ($P < 0.001$) reduced both phases of pain response with a maximum inhibition of 86.62 ± 3.35% and 95.91 ± 2.67%, respectively (Fig. 5).

Dose-response curves plotted using non-linear regression estimated the ED_{50} values of EthOL and EthYL (Fig. 6) as 72.67 ± 1.22 mg/kg and 48.19 ± 1.17 mg/kg respectively for phase 1. Similarly, phase 2 ED_{50} values calculated for EthOL and EthYL were 74.12 ± 1.11 mg/kg and 59.79 ± 1.08 mg/kg respectively. EthYL was 1.5 times more potent than EthOL in the first phase and 1.23 times more potent than EthOL in the second phase.

3.5. Mechanism of analgesic effect

Figs. 7–10 show the effect of pre-treatment of mice with naloxone (2 mg/kg, i.p.), atropine (5 mg/kg, i.p.), nifedipine (10 mg/kg, *p.o.*), glibenclamide (8 mg/kg, *p.o.*), L-NAME (10 mg/kg, i.p.) and yohimbine (3 mg/kg, *p.o.*) on the anti-nociceptive activities of EthOL, EthYL and morphine. Both phase 1 and phase 2 anti-nociceptive effects of EthYL, EthOL, (200 mg/kg, *p.o.*) and morphine were reversed by pre-treatment of mice with naloxone and L-NAME. In addition, phase 2 of atropine pre-treatment reversed the antinociceptive activity of EthYL but not EthOL. Pre-treatment with all the antagonists reversed the anti-nociceptive activity of morphine in both phases of the formalin test (Fig. 10).

3.6. Histopathological evaluation

Fig. 11A–I reveals the histoarchitecture of the dermis from the right hind paw of the mice at a total magnification of ×100, using the H&E stain. Section from the normal control group (Fig. 11A), has irregularly arranged loose upper and dense lower dermal connective tissue layer just below the epidermis. Tissue fibres looks compact with no extravasation of leucocytes. In contrast, the negative control group (Fig. 11B) shows severe leucocytic infiltration accompanied with dilation of blood vessels. Leucocytosis are mostly evident in perivenular areas. Some vessels show congestion accompanied with leucocytic pavementing and margination on endothelial walls. Edema is present, but masked by erythrocytic infiltrate in congested areas. In the positive control group (Fig. 11C), sections show mild leucocytic infiltrate, with noticeable signs of edema. The reference drug (Morphine) seems to counteract the leucocytic transmigratory pathway, hence reducing the inflammatory cell infiltrate.

For the lower dose of the extracts, sections from the 50 mg/kg EthYL (Fig. 11D) reveals areas of focal leucocytic infiltrates devoid of congestion, with the 50 mg/kg EthOL (Fig. 11E), showing diffused leucocytic infiltrates accompanied with congestion and mild edema. The lesions in Fig. 11E is comparably lower than the ones found in the

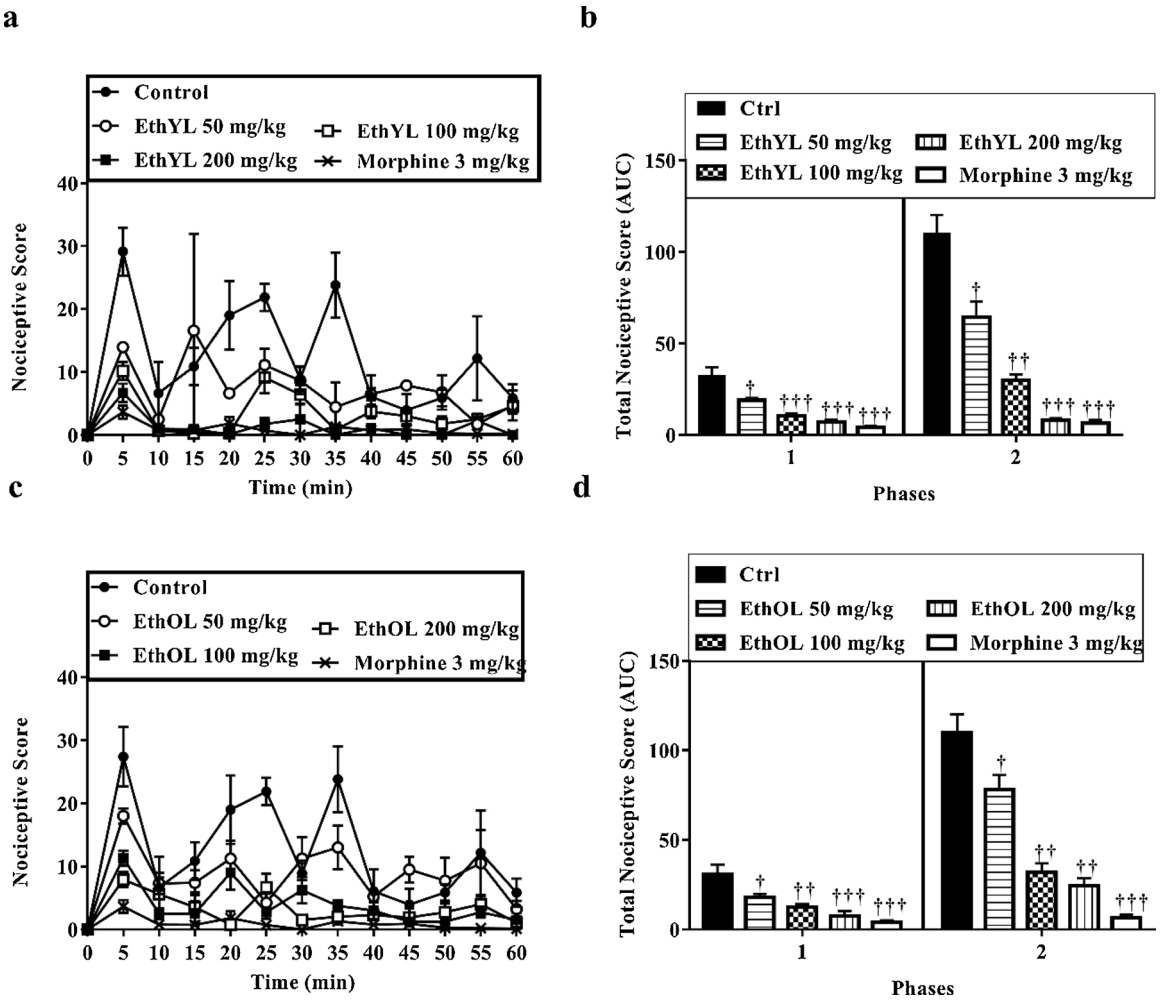

Fig. 5. Effect of EthOL (50, 100 and 200 mg/kg, *p.o.*), EthYL (50, 100 and 200 mg/kg, *p.o.*), and morphine (3 mg/kg, i.p.) on the time-course curves (a and c) and the total nociceptive score (calculated as AUCs) (b and d) in formalin-induced nociceptive test in mice. Each data represents the mean ± S.E.M (n = 5). The symbol † indicate significance levels compared to respective controls: $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger}P < 0.05$ (one - way ANOVA followed by Newman Keuls' *post hoc*).

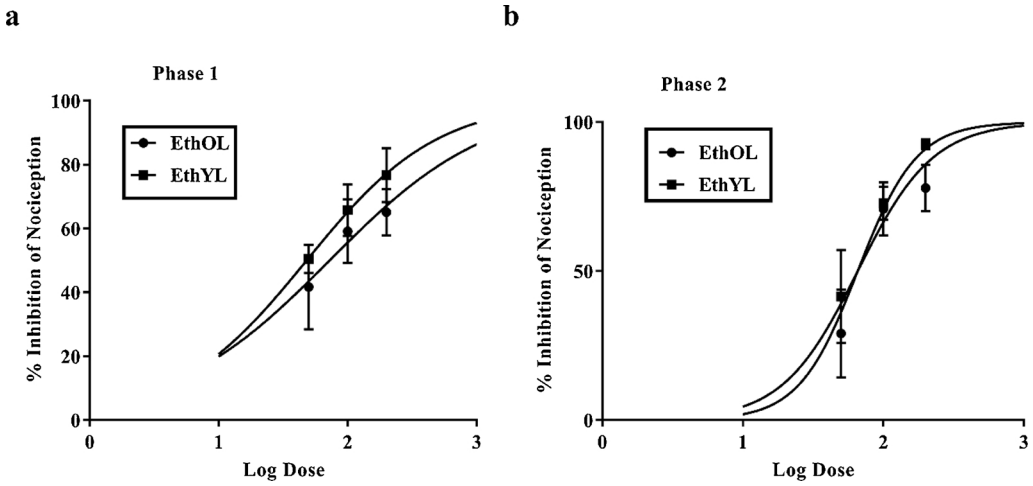

Fig. 6. Dose-response curves of EthOL (50, 100 and 200 mg/kg, *p.o.*) and EthYL (50, 100 and 200 mg/kg, *p.o.*) in phase 1(a) and phase 2(b) of formalin-induced nociception test in mice.

Fig. 11B (negative control group). Furthermore, sections from the mice dosed with 100 mg/kg EthYL (Fig. 11F), shows mild edema with tissue architecture comparable to the normal control group. The 100 mg/kg EthOL (Fig. 11H) on the other hand, shows mild leucocytosis accompanied by focal congestion in vessels. For the high dose (200 mg/kg) of both extracts, sections from the EthYL group shows tissue architecture comparable to the normal control group; thus, indicative of high anti-inflammatory activity. However, the EthOL did not show similar anti-inflammatory effect; it reveals focal areas of mild leucocytosis accompanied with edema.

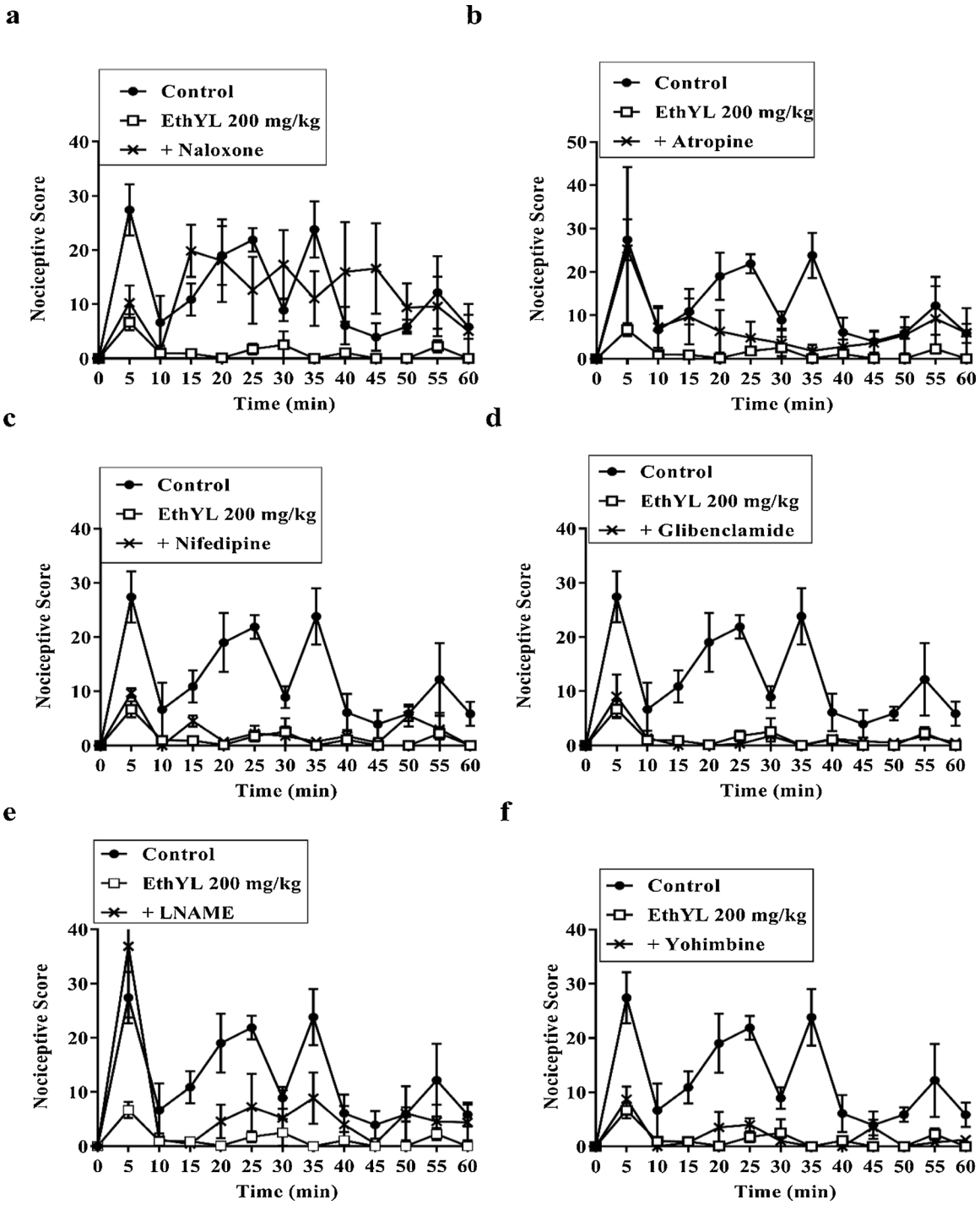

Fig. 7. Effect of pre-treatment of mice with (a) naloxone (2 mg/kg i.p.), (b) atropine (5 mg/kg, i.p.), (c) nifedipine (10 mg/kg, *p.o.*), (d) glibenclamide (8 mg/kg, *p.o.*) (e) L- NAME (10 mg/kg, i.p) and (f) yohimbine (3 mg/kg, *p.o.*), on the anti-nociceptive profile of EthYL, (200 mg/kg, *p.o.*) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M.

For histological evaluation of dermal mast cells (MC) in association with leucocytic infiltration (Fig. 12A–I), the routine H&E stain was employed once more, and analysed at a total magnification of ×400. These connective tissue MCs in the dermal areas were mostly found around the perivascular or venular areas. These cells, which are residential mononuclear immune cells could be differentiated from extravasated leucocytes by their large and highly conspicuous eosinophilic cytoplasm (Fig. 12). MCs could be seen in the intact or non-activated state in the normal control group (Fig. 12A), with no association with peripheral leucocytic infiltrates. The negative control group (Fig. 12B) on the other hand, shows degranulation of MCs, associated with high infiltration of polymorphonuclear leucocytes (PMNL); mostly multilobed neutrophils (predominant cells in acute inflammation). Signs of high inflammatory exudate (edema) are also evident. The positive control group (Fig. 12C) had few moderately degranulated MCs and also non-activated MCs, accompanied with mild leucocytic infiltrate. Sections from 50 mg/kg EthYL (Fig. 12D) group had few PMNL as compared with Fig. 12C. It also shows very few intact and moderately degranulated MCs, with signs of exudation (edema). The 50 mg/kg EthOL group (Fig. 12E), reveals sections that show

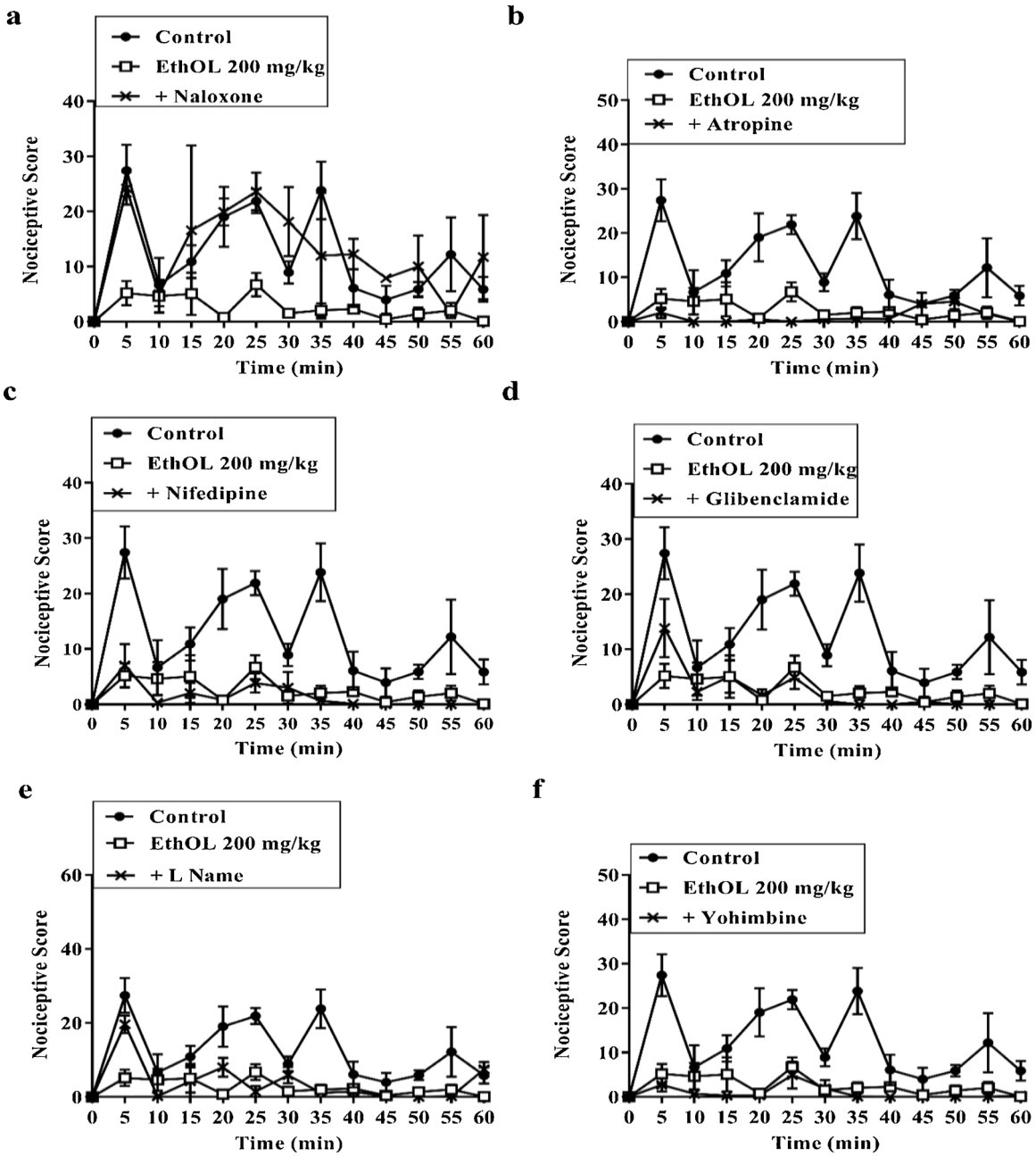

Fig. 8. Effect of pre-treatment of mice with (a) naloxone (2 mg/kg i.p.), (b) atropine (5 mg/kg, i.p.), (c) nifedipine (10 mg/kg, *p.o.*), (d) glibenclamide (8 mg/kg, *p.o.*) (e) L- NAME (10 mg/kg, i.p) and (f) yohimbine (3 mg/kg, *p.o.*), on the anti-nociceptive profile of EthOL, (200 mg/kg, *p.o.*) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M.

moderate PMNL infiltrate with few moderately degranulated MCs, with signs of edema. Fig. 12F (100 mg/kg EthYL) demonstrates the presence of activated MCs, with very few patchy PMNL infiltrate, devoid of edema. Similarly, the 100 mg/kg EthOL (Fig. 12G), shows mild degranulated MCs accompanied by moderate leucocytic infiltrate, with no sign of edema. For the high dose (200 mg/kg) of the extracts, section from the EthYL group (Fig. 12H), shows intact MCs with very few PMNL. Tissue consistency in this group is likened to that of the normal control group. Sections from its counterpart (EthOL) as shown in Fig. 12I, demonstrates the presence of both intact and mildly degranulated MCs, with moderate infiltration of PMNL.

Based on the degree of degranulation, using toluidine blue stain, four main types of MCs were identified during the inflammatory condition. These are; (a) Intact or non-activated MCs: Deep blue orthochromatic staining with conserved cell membrane without extrusion of granules, and having pale metachromatic nuclei, (b) Activated MCs: Amorphous shape with no sign of degranulation, but absence of visible pale metachromatic nuclei, (c) Moderate/mild degranulation: MCs from which some granules have been released, but outline of cell membrane retained (d) Severe degranulation: Cytoplasmic granules of MCs is widely spread, accompanied by total distortion of the outline of its cell membrane. These have been reported previously [41,42]; excluding 'b' (identified by this current research).

Using the criteria described above, Fig. 13A (normal control group) shows sections populated with intact MCs. The negative control (Fig. 13B) reveals MCs with severe degranulation. The positive control (Fig. 13C) on the other hand, demonstrates population of activated and mildly degranulated MCs. Thus, good MC stabilizing activity of the reference drug (Morphine).

Sections from both low dose (50 mg/kg) groups, show mild to

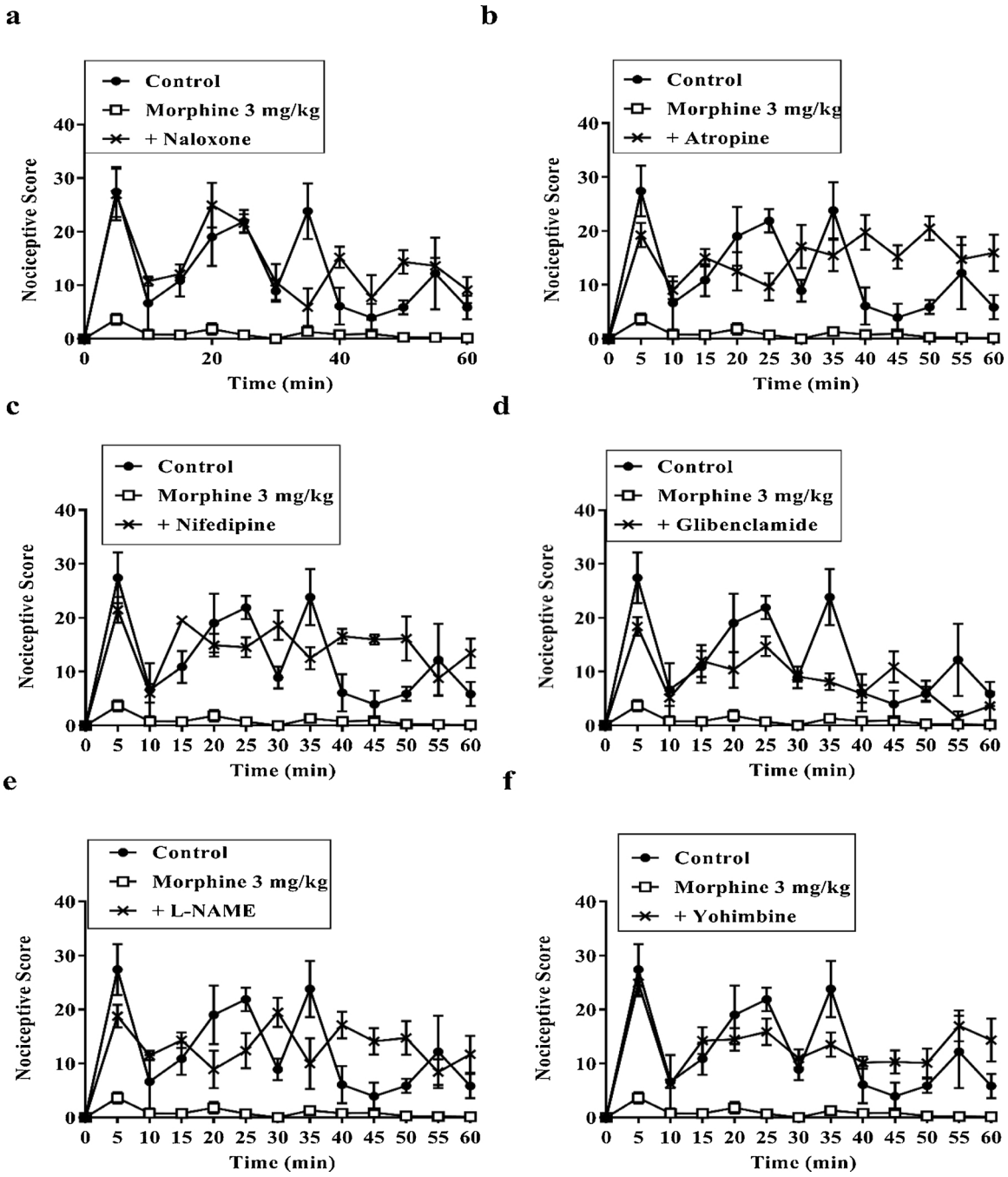

Fig. 9. Effect of pre-treatment of mice with (a) naloxone (2 mg/kg i.p.), (b) atropine (5 mg/kg, i.p.), (c) nifedipine (10 mg/kg, *p.o.*), (d) glibenclamide (8 mg/kg, *p.o.*) (e) L- NAME (10 mg/kg, i.p) and (f) yohimbine (3 mg/kg, *p.o.*), on the anti-nociceptive profile of morphine (3 mg/kg, i.p.) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M.

severe degranulation of the MCs, with the 50 mg/kg EthYL (Fig. 13D) showing mild degranulation, and its counterpart, EthOL (Fig. 13E), exhibiting moderate to severe degranulation of the MCs. Histological section from the 100 mg/kg EthYL group (Fig. 13F) shows mild and activated MCs, with the old counterpart (Fig. 13G) showing predominantly MCs with mild degranulation. Thus, both 50 and 100 mg/kg EthYL show higher MC stabilizing effect than their corresponding EthOL (50 and 100 mg/kg) groups respectively. Fig. 13H (200 mg/kg EthYL) shows intact and activated MCs with no sign of degranulation. Fig. 13I (200 mg/kg EthYL) on the other hand, shows intact, activated and moderately degranulated MCs.

From the histological evaluation of the MCs made, the EthYL at all doses, demonstrates a higher stabilizing activity than EthOL. And since nerve endings reside in the dermal region and responds to granules released by MCs during inflammatory conditions [41], this may partly explain the relatively reduced nociceptive sensitisation and the high anti-inflammatory activity as compared to the negative control groups. Thus, from the histopathological results, the high dose (200 mg/kg) of the EthYL, showed the highest stabilizing effect of the connective tissue MCs and demonstrated minimal perivascular PMNL infiltrate within its tissue section.

4. Discussion

This study was conducted to ascertain the therapeutic usefulness of young and old leaves of *V. amygdalina* in managing pain, edema, and

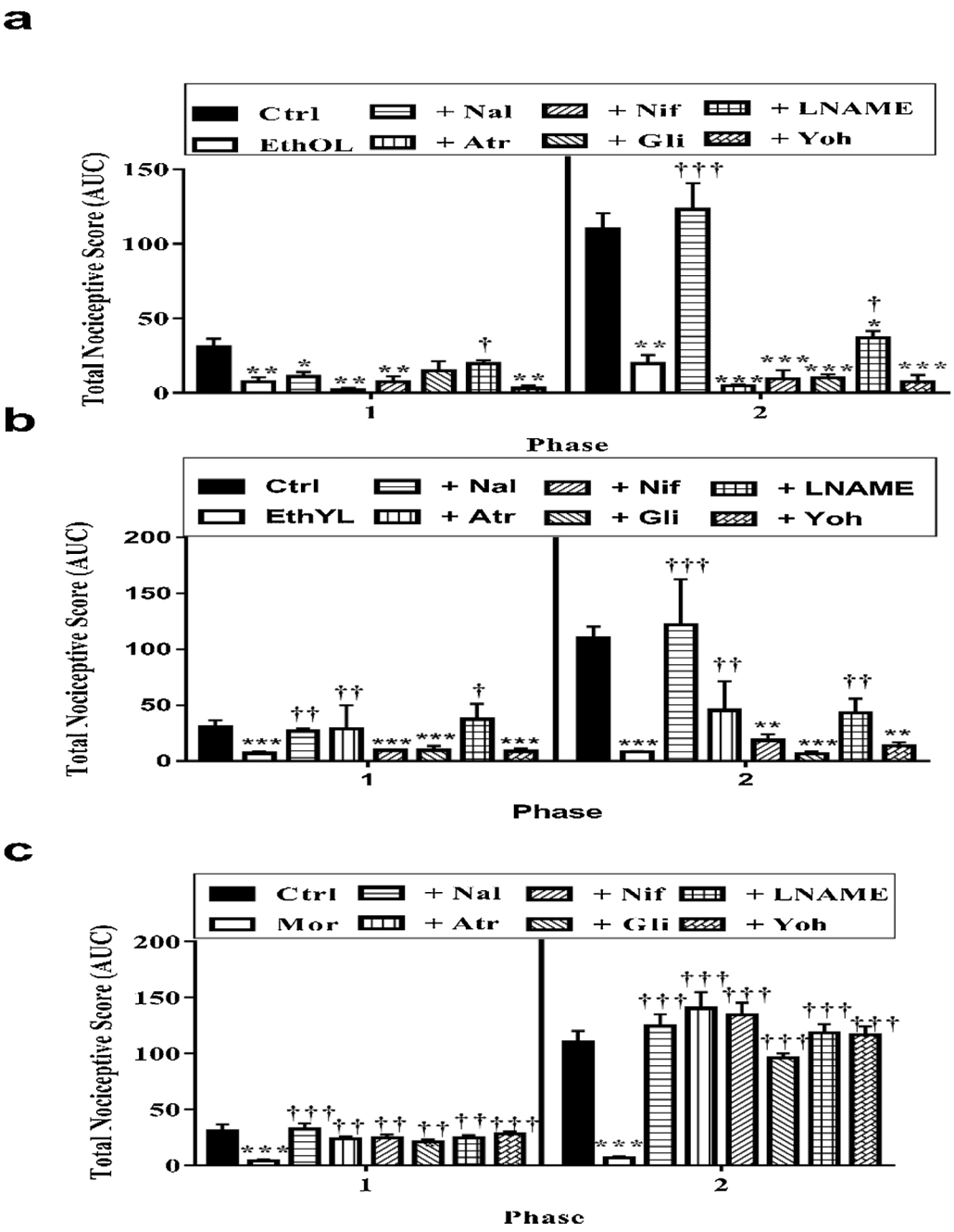

Fig. 10. Effect of pre-treatment of mice with naloxone (2 mg/kg i.p.), atropine (5 mg/kg, i.p.), nifedipine (10 mg/kg, *p.o.*), glibenclamide (8 mg/kg, *p.o.*), L-NAME (10 mg/kg, i.p), yohimbine (3 mg/kg, *p.o.*), on the anti-nociceptive profile of (a) EthYL (200 mg/kg, *p.o.*), (b) EthOL (200 mg/kg, *p.o.*) and (c) morphine (3 mg/kg, i.p.) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M. compared to respective vehicle-treated controls; $^{***}P < 0.001$,$^{**}P < 0.01$ and $^{*}P < 0.05$ compared to vehicle treated control and $\dagger\dagger\dagger P < 0.001$, $\dagger\dagger P < 0.01$ and $\dagger P < 0.05$ compared to either EthYL (200 mg/kg, *p.o.*), EthOL (200 mg/kg, *p.o.*) or morphine 3 mg/kg treated control (all one-way ANOVA followed by Newman-Keuls' *post hoc*).

pyrexia associated with acute inflammation. Baker's yeast was used to induce experimental pyrexia, while carrageenan was used to induce experimental edema and cold allodynia in Sprague-Dawley rats. Tail immersion was also used to induce thermal hyperalgesia which increased pain sensitivity [20]. Further assessment of pain was done using the formalin-induced nociceptive model and possible mechanism of action of the extracts evaluated.

In the current study, we compared the efficacy of EthYL and EthOL as anti-inflammatory agent and analgesic using different pain models. Intraplantar injection of carrageenan is known to induce the release of inflammatory mediators such as prostaglandins and other cyclo-oxygenase (COX) products [43,44], Non-steroidal anti-inflammatory drugs (NSAIDS) are known to mediate their pharmacological actions through the inhibition of cyclo-oxygenase (COX) enzymes [45]. Both EthYL and EthOL (200 mg/kg) significantly inhibited the paw edema in the 4th hour post- carrageenan induction. These results suggest that both EthYL and EthOL extracts might act as peripheral analgesics, altering the local reaction caused by the release of pain and inflammatory mediators.

We then examined the effect of these extracts using the cold allodynia. In this test, hyperalgesia was induced by intraplantar injection carrageenan into the rats' right hind paw and the same paws were inserted into cold water (4 °C). Allodynia is a hallmark symptom of neuropathic pain, which occurs when non innocuous stimuli stimulate Aß-fibers or low-threshold Aδ- and C- nociceptive fibers [46,47]. Carrageenan pre-treatment is known to initiate an acute inflammatory response which lowers pain threshold of nociceptors within the periphery leading to central sensitization and hypersensitivity [48]. However, treatment with both EthYL and EthOL significantly inhibited the cold allodynia.

Tail immersion test is another important acute pain model used to screen for centrally acting analgesics and opioid receptor agonist. Hyperalgesia measured in the tail immersion model involves supraspinal and spinal structures of the central nervous system (CNS) [49], and is selective for compounds that act by the activation of opioid

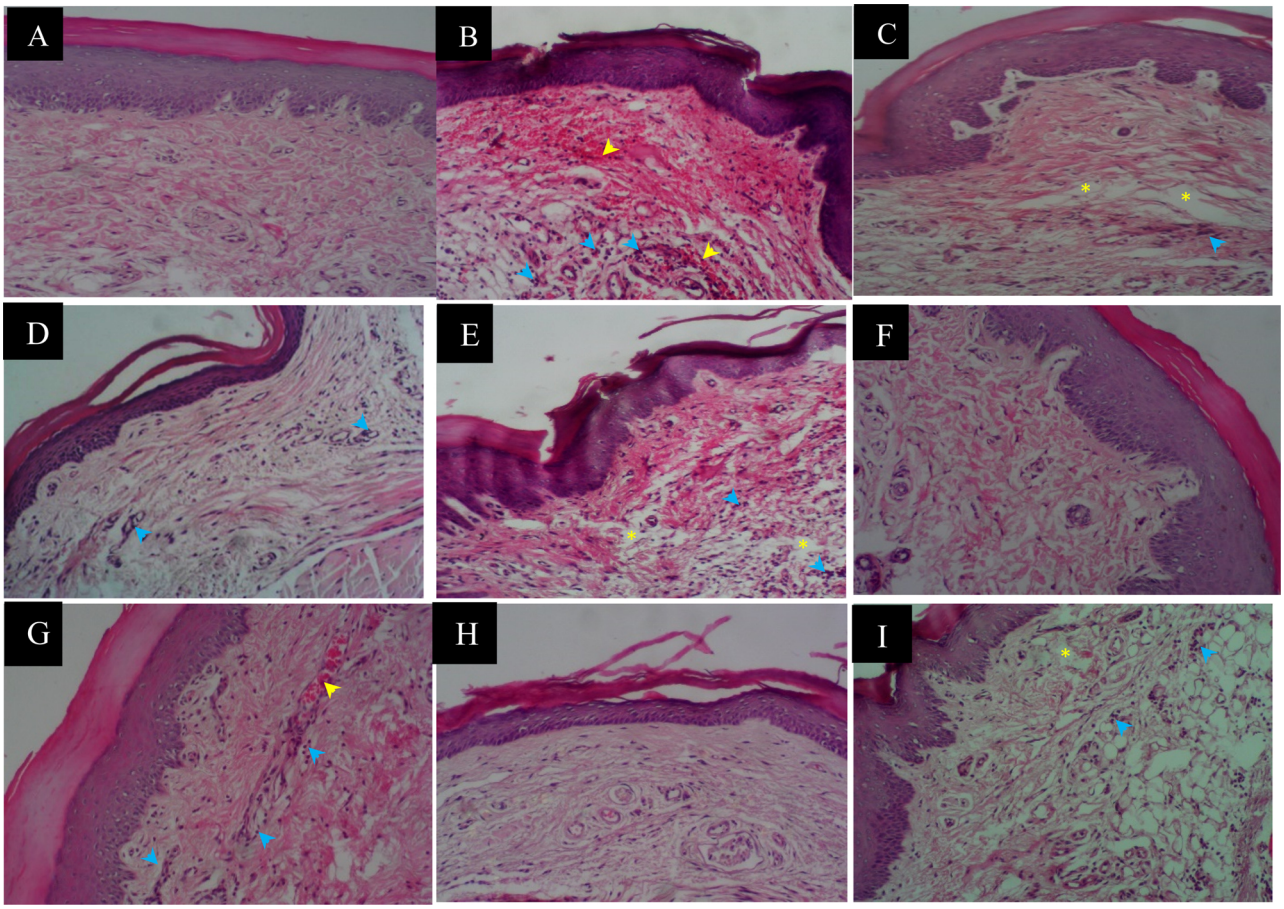

Fig. 11. Photomicrograph of tissue section of the right hind-paw in formalin induced nociceptive test (H&E ×100). (A) Normal control: the dermis appears normal (B) Negative control: the leukocyte infiltration is severe (blue arrowhead), dilation and congestion of vessels are evident (yellow arrowhead) (C) Positive control: shows very mild levels of leukocytic infiltrate (blue arrowhead), accompanied by edema (yellow asterisks) (D) EthYL 50 mg/kg: section depicts moderate leukocytosis (blue arrow head) (E) EthOL 50 mg/kg: leukocytosis (blue arrow head) and mild edema (yellow asterisks) present (F) EthYL 100 mg/kg: mild edema present. Tissue consistency is fairly normal (G) EthOL 100 mg/kg: section shows mild leukocytosis (blue arrowhead) accompanied by congestion (yellow arrowhead) (H) EthYL 200 mg/kg: Tissue architecture comparable to the normal control (I) EthOL 200 mg/kg: leukocytosis (blue arrowhead) is mild accompanied with mild edema (yellow asterisk) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

receptors. The posterior (dorsal) horn of the spinal cord is endowed with several neurotransmitters and receptors, such as substance P, neuropeptide Y, inhibitory amino acid, nitric oxide (NO), endogenous opioids, bradykinin and monoamines, which are major targets for pain medications [50]. Compounds that can inhibit thermal hyperalgesia interact with κ-opioid receptors and produce analgesia by causing hyperpolarization of interneurons within the dorsal spinal cord and depressing the release of transmitters such as enkephalin, serotonin, or norepinephrine, and specific receptors located on the nociceptive fibers that transmit pain sensation to the higher centers. Phytopharmaceuticals can also interact with μ-opioid receptors located in the supraspinal structures and activate the supraspinal system to release endorphins that inhibit the transmission of pain signals by these fibers [51,52]. In contrast, some non-steroidal anti-inflammatory drugs (NSAIDs) such as Diclofenac act by inhibiting both peripheral and central pain and is used as a reference drug in this study [53,54]. Both EthYL and EthOL of VA increased the latency period while decreasing thermal hyperalgesia, similar to Diclofenac.

Formalin test is also a highly predictive pain model which mimics acute and clinical pain [47]. This model was used to assess the mechanism of action of the EthYL and EthOL extracts [37,38]. EthYL extracts produced a significantly higher anti-nociceptive effect in both neurogenic and inflammatory phases compared to the EthOL at all dose levels in a dose-dependent pattern. This agrees with the analgesic effect observed in the tail immersion and cold allodynia models.

Further evaluation for the mechanism of analgesic effect was carried out and observed results showed that, naloxone, a nonselective opioid antagonist, significantly reversed the anti-nociceptive effect of both EthYL and EthOL in both phases of the formalin-induced nociception; suggesting a possible opioidergic involvement in their mechanisms of actions. Also, a nitric oxide (NO) synthase inhibitor (L-NAME) significantly reversed the anti-nociception produced by both extracts. Nitric oxide is known to play a complex part in transmission of nociceptive signals peripherally and centrally [55,56]. The anti-nociceptive effect of EthYL and EthOL could possibly involve nitric oxide cyclic GMP pathway. Additionally, atropine (nonselective muscarinic receptor antagonist) significantly reversed the anti-nociceptive effect of EthYL, but not EthOL. These results implicate the involvement of muscarinic cholinergic system in the mechanism of action of EthYL.

In vitro studies have demonstrated the anti-inflammatory activity of flavonoids by the inhibiting the synthesis of various pro-inflammatory mediators such as eicosanoids, cytokines, adhesion molecules and C-reactive protein [57]. Alkaloid [58], tannin [59], flavonoid [60] and saponin fractions [61] have been reported to be responsible for the analgesic ability of medicinal plants. The analgesic activity of saponins and flavonoids have been identified *via* inhibition of the synthesis and activities of enzymes involved in the production of inflammatory-related chemical mediators, such as prostaglandins [62], while alkaloids inhibit pain *via* opioid receptors in the CNS [63]. Observed results shows that EthYL has more potent anti-inflammatory and analgesic

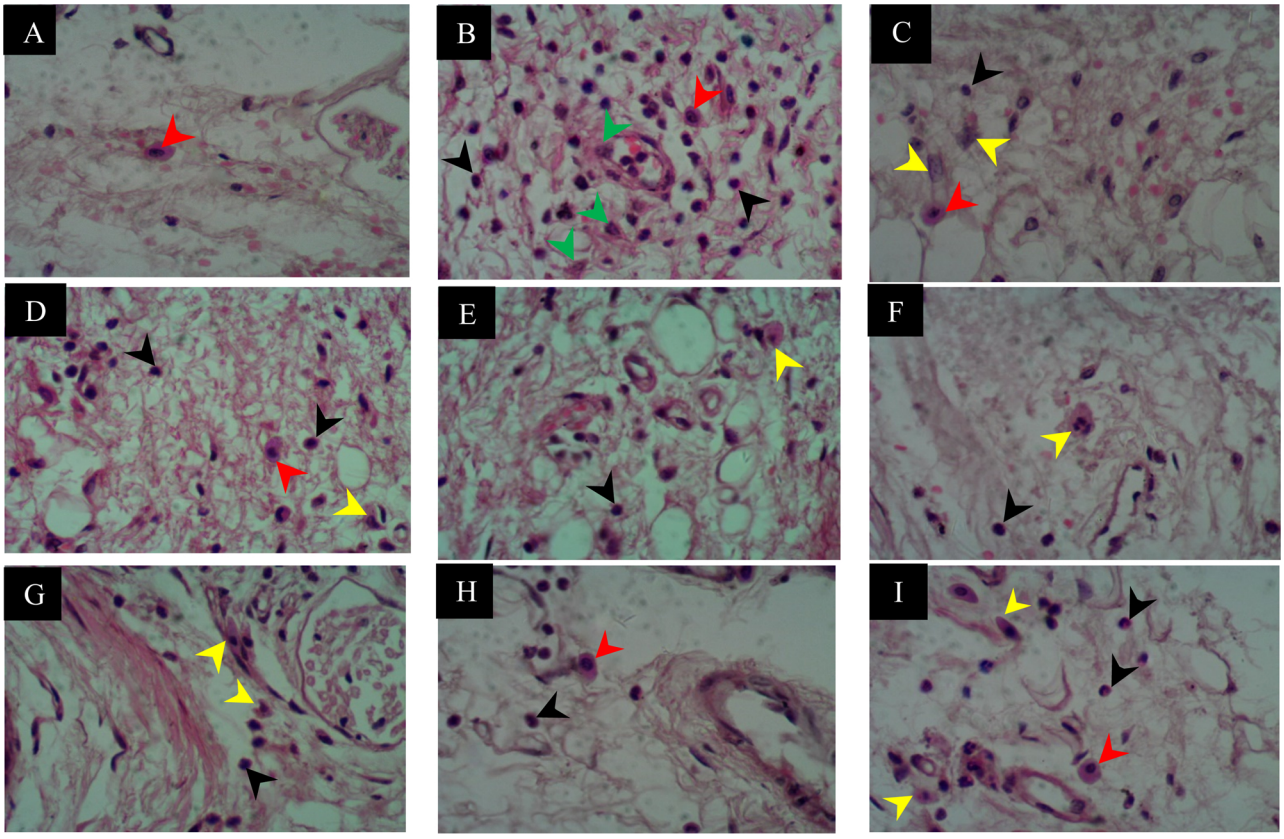

Fig. 12. Photomicrograph of tissue section of the right hind paw in formalin induced nociceptive test (H&E ×400). It depicts immune cell infiltrates in association with Mast cell (MC) degranulation dynamics. Neutrophilic/leucocytic immune infiltrate: *Black arrowhead*; Non-activated/intact MCs: *Red arrowhead*; Mild to moderate degranulation: *Yellow arrowhead*; Severe degranulation: *Green arrowhead*. **(A)** Normal control: section shows intact MC devoid of neutrophilic infiltrates (B) Negative control: severe degranulation of MCs, accompanied by high leucocytic infiltrate (C) Positive control: shows few moderately degranulated and intact MCs with very mild leucocytosis (D) EthYL 50 mg/kg: few neutrophilic infiltrate. (E) EthOL 50 mg/kg: show moderate neutrophilic infiltrate. (F) EthYL 100 mg/kg: activated MC, with very few neutrophilic infiltrates (G) EthOL 100 mg/kg: sections show moderate neutrophilic infiltrates. (H) EthYL 200 mg/kg: section shows intact or non-activated MC with very few neutrophilic infiltrate (I) EthOL 200 mg/kg: both intact and moderately degranulated MCs, with moderate leucocytosis (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

activity, relative to the EthOL. Difference in the amount of the bioactive compounds and antioxidant capacities may play a significant role in the results realized.

Also, high levels of TAC are reported to protect tissues from injury caused by oxidation reactions, reducing inflammation-induced pain [64]. High levels of TAC was observed in YL, relative to OL, and similar results were reported in a previous study [15]. This could be responsible for the anti-inflammatory activity of the YL by reducing the oxidative stress induced by reactive oxygen species (ROS) during the inflammatory process [65], and could be partly attributed to the analgesic activity of this group.

MCs are main effector cells of allergic and other inflammatory reactions [66,67]. They are predominantly found in the dermis near blood vessels, nerves or the basement membrane of endothelial cells. They possess cytoplasmic granules containing histamine, serotonin, proteases and various cytokines including TNF, IL, fibroblast growth factors (FGF), and epithelial growth factor (EGF), that mediates inflammation [68,69]. This implies that the inhibition of MC activity can subsequently lead to inhibition of inflammation. Observed results from histopathological analysis, showed severe MC degranulation in the negative control accompanied by vasodilatation and high leukocytosis, and lowest in both 100 and 200 mg/kg of both EthYL and EthOL extracts. It was moderate in those treated with aspirin (positive control), whiles 50 mg/kg of both extracts showed significant MC degranulation.

Most importantly, EthYL at all doses (50–200 mg/kg), showed decrease levels of MC degranulation as compared with their old counterparts (50–200 mg/kg EthYL). This could be due to the higher antioxidant effect in EthYL as shown in the current results. This is in accordance with previous research that demonstrated the antioxidant stabilizing activity of drugs and extracts on MCs [41,70,71]. Taken together, this suggest that, EthYL was able to reduce or inhibit the transmigration and trafficking of pro-inflammatory cells such as neutrophils to site of formalin induction, partly by the stabilization of residential MCs in a more efficient manner, as compared to EthOL.

The subcutaneous injection of *Saccharomyces cerevisae* suspension introduces exogenous pyrogens that induce the production of pro-inflammatory cytokines such as interleukins 1β and 6 (IL-1β, IL-6), interferon (IF-α) and tumor necrosis factor (TNF) [8]. These cytokines enter hypothalamic circulation and stimulate the release of local prostaglandins, mainly Prostaglandin E2 (PGE2) which elevate the hypothalamic thermal set point beyond normal temperatures [72,73].

Phytochemical screening of VA revealed the presence of steroids, tannins, triterpenoids, flavonoid, and glycosides [15], which have been demonstrated to be responsible for the antipyretic abilities of many medicinal plants [74–76], including VA [61,77]. Our results demonstrate that both EthYL and EthOL extracts exhibit antipyretic activity. However, a 2-fold increase in the anti-pyretic activity was observed in the EthYL extracts, relative to the EthOL. This once more, could be attributed to the difference in the amount of the bioactive compounds and antioxidant prowess of the two extracts.

The cumulative effect of other unscreened novel bioactive compounds present in the EthYL, acting in a synergistic fashion (other than saponins only), may be partially responsible for the potent anti-inflammatory, analgesic and antipyretic activity of the EthYL realized in

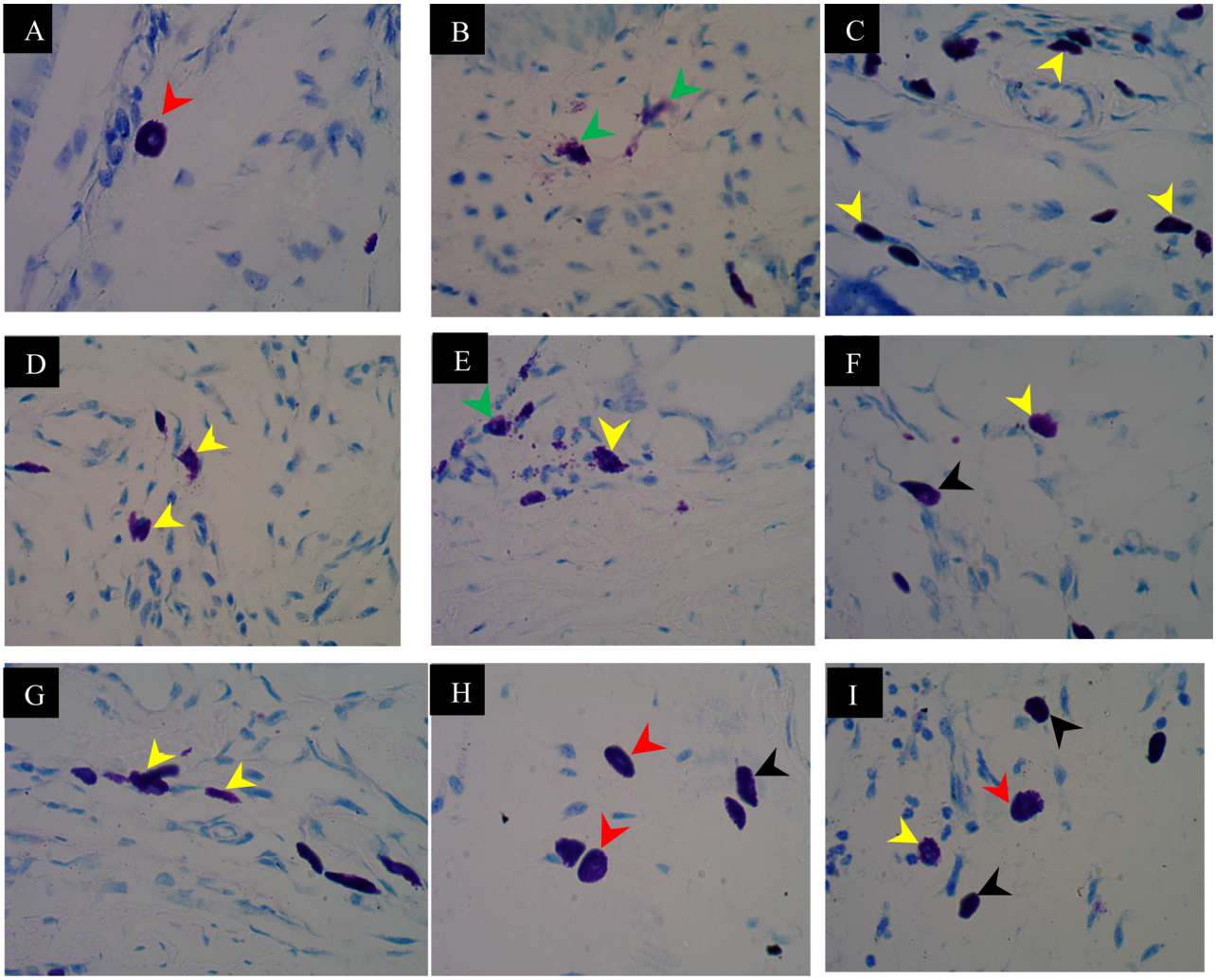

Fig. 13. Photomicrograph of tissue section of the right hind paw in formalin induced nociceptive test (Toluidine blue ×400). It shows mast cell (MC) activation and degranulation dynamics. Non-activated/intact MCs: *Red arrowhead*; Activated: *Black arrowhead*; Mild to moderate degranulation: *Yellow arrowhead*; Severe degranulation: *Green arrowhead*. (A) Normal control: section shows intact MC (B) Negative control: severe degranulation of MCs (C) Positive control: shows activated and moderately degranulated MCs (D) EthYL 50 mg/kg: mild degranulation in this group (E) EthOL 50 mg/kg: sever to moderate degranulation. (F) EthYL 100 mg/kg: shows activated MCs (G) EthOL 100 mg/kg: reveals mild degranulation (H) EthYL 200 mg/kg: MCs in this section could be likened to the normal control (I) EthOL 200 mg/kg: both intact, activated and moderately degranulated MCs (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

this study.

Thus, it will be interesting for further studies to be carried out to unravel these bioactive compounds in EthYL.

5. Conclusion

Young and Old leaves of *V. amygdalina* ameliorate pyrexia, cold allodynia, thermal hyperalgesia, and edema associated with acute inflammation. Both extracts ameliorated the neurogenic and inflammatory phases of the formalin induced nociception, with the EthYL being slightly more potent than EthOL. The mechanism of action of both EthYL and EthOL possibly involves the opioidergic and nitric oxide cyclic GMP pathways. EthYL may have an additional mechanism which implicates the muscarinic cholinergic system.

Indeed, the EthYL shows promise as a potent agent for conditions associated with inflammation (pain, swelling and fever), and clearly justifies the indigenous use of these young leaves for the treatment of the aforementioned conditions in the southern part of Ghana.

Also, considering the days used by the young leaves to reach 5.10 cm (lower limit by length), plus the estimated 21 days, we propose that VA leaves should be at most, 30 days old to be considered as young vegetative leaves, for its usage as potent antipyretic, analgesic and anti-inflammatory agents.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.12.147.

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