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Original Research Article

Analgesics from *Lonchocarpus eriocalyx* Harms

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ABSTRACT

Four lupane-type terpenoids, namely lupeol (**1**), friedelin (**2**), stigmasterol (**3**), and stigmasterol-3-*O*-glucoside (**4**) were isolated from the ethyl acetate extract of leaves of *Lonchocarpus eriocalyx* Harms. These compounds were obtained by extensive silica gel chromatography and their structures elucidated by 1D and 2D nuclear magnetic resonance (NMR) as well as comparison with literature data. The ethyl acetate, dichloromethane and methanol extracts (100 mg/Kg) had a pretreatment latency of 3.1±0.15, 3.0±0.01 and 3.5±0.12 at the zero minute. The post latency of 6.4±0.13 was observed for ethyl acetate at 30 minutes which confirmed its effectiveness to halt pain, while compounds **1** and **2** had pretreatment latency of 3.1±0.12 and 3.2±0.12, respectively. The isolated compounds from this medicinal plant along with their analgesic activity have been reported for the first time.

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1. Introduction

Medicinal plants are of paramount interest in different scientific disciplines (Mohammadhosseini et al., 2019). They exert a variety of promising impacts on human beings (Mohammadhosseini, 2017; Mohammadhosseini et al., 2017). Many herbal plants are widely used in traditional folk medicine of most countries worldwide (Frezza et al., 2017; Venditti and Bianco, 2018; Venditti et al., 2018).

An analgesic or painkiller is any member of the group of drugs which is often used to achieve analgesia/relief from pain. The commonly used analgesic drugs are aspirin, paracetamol and morphine among others (Shashank et al., 2013). Response to pain in animals can be investigated by applying unpleasant stimuli such as (i) thermal like radiant heat as a source of pain, (ii) chemical irritants such as acetic acid and (iii) physical pressure like tail compression (Kulkarni, 2003). Today, many herbal preparations are being prescribed as analgesics as well as anti-inflammatory agents and in the past decade, there has been a resurgence of interest in traditional systems of medicine which has become a topic of global importance (Adair, 2001). It is estimated that in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants in pain relief implying that phytomedicines have continued to maintain popularity (Smet, 1997). Indeed, many important drugs in the market have been obtained directly or indirectly from natural sources, i.g. morphine, pilocarpine, digitalis, quinine, artemisinin among others (Cragg et al., 1997; Verpoorte, 1998; Strohl, 2000). *Lonchocarpus eriocalyx* Harms belonging to the family Fabaceae commonly known as Leguminosae is frequently used to control fever, headache and diarrhea and also as an effective insecticide remedy (Ceres et al., 1981).

The genus *Lonchocarpus* is a rich source of many different classes of compounds including terpenoids, aurones, chalcones, dibenzoylmethane derivatives, pterocarpans, rotenoids, flavanones, flavanols, flavans, flavones, flavonols, and isoflavones (Cassidy et al., 2011). According to the literature, the crude extract from the root bark of *L. eriocalyx* exhibited antiplasmodial activity

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against *Plasmodium ovale* (Tuwei, 2006). In addition, chromatographic separation of the extract from the plant yielded lupeol (**1**), which showed remarkable antiplasmodial activity (Yenesew et al., 2003). In this study, we report the isolation of lupeol (**1**), friedelin (**2**) stigmasterol (**3**), and stigmasterol glucoside (**4**), of which the compounds **2** and **3** are reported for the first time from this plant. The analgesic properties of the isolated compounds are also evaluated.

2. Experimental

2.1. Reagents, chemicals and fine consumables

Melting points were determined using Gallenkamp melting point apparatus and were uncorrected. The NMR data were measured in $CDCl_3$ and $CDCl_3$-DMSO-d_6 on Bruker AM 300 spectrometer operating at 300 and 75 MHz, for proton and carbon experiments, respectively. TMS was used as an internal standard. The mass spectral data were obtained using a Varian MAT 8200 A instrument. Column chromatography was performed using silica gel 60 (0.063-0.200 mm, Merck), while thin layer chromatography (TLC) was performed using silica gel 60 F_{254} (Merck) pre-coated plates. All solvents used were of analytical reagent grade and used without further purification.

2.2. Collection of plant materials

Leaves of *Lonchocarpus eriocalyx* Harms were collected from Embu-Mbeere (Lat: 0.5833º S and Long: 37.6333º E) where it naturally occurs. The plant materials were authenticated at the Herbarium of the Museums of Kenya where voucher specimens are preserved (Refernce No.: LE/58/2013).

2.3. Extraction and isolation

The air-dried and pulverized leaves (1.5 kg) of the plant were soaked sequentially in *n*-hexane (3 x 3 L), EtOAc (3 x 3L) and MeOH (3 x 3L), each lasting four days at room temperature. The extracts were separately filtered and evaporated under reduced pressure to afford yellowish (1 g), brown (106 g) and reddish-brown (2 g) extracts of *n*-hexane, EtOAc and MeOH, respectively. The extracts were kept at 4 ºC for further phytochemical and analgesic activity studies.

2.4. Fractionation of EtOAc extract

Ethyl acetate extract (30 g) was adsorbed onto silica gel and subjected to column chromatography (2.5 x 60 cm, SiO_2 300 g, pressure≈1 bar) starting with pure n-hexane, then using *n*-hexane-CH_2Cl_2 gradient (increment 10%) up to 100% CH_2Cl_2. The elution process was finalized using CH_2Cl_2-ethyl acetate gradient (increment 10% up to 100% ethyl acetate, collecting 200 fractions of 20 mL (each). The obtained fractions were merged into five sub-fractions (I-V) as determined by TLC profiles [solvent systems: *n*-hexane-CH_2Cl_2 (1:3, 1:2) and CH_2Cl_2-MeOH, 95:5 and 90:10)]. The sub-fraction I (fractions 1-10) showed no spot and solvent was recovered. Sub-fraction II (fractions 15-60, 20 g) gave a single spot R_f 0.83 on TLC (eluent: *n*-hexane-EtOAc, 2:3) which upon evaporation of solvent followed by crystallization in CH_2Cl_2-MeOH of ratio 1:1 mixture afforded compound **1** as white needle-like crystals (55 mg). Sub-fraction III (fractions 61-86, 5 g) showed two spots of R_f values 0.82 and 0.62 (eluent: *n*-hexane-EtOAc, 2:3) which upon repeated column chromatographic separation afforded a further **1** (45 mg) and **2** (50 mg), respectively. Sub-fraction IV (fraction 93-103, 7.4 g) showed two major spots R_f 0.48 and 0.65 (eluent: CH_2Cl_2-MeOH, 97:3) and upon evaporation of solvent, followed by crystallization gave compounds **3** (64 mg) and **4** (35 mg), respectively. Fractions 104-180 constituted sub-fraction V (5 g) and were further purified by medium pressure chromatography (2.5 x 50 cm, SiO_2 150 g, pressure≈1 bar) to give further compounds **3** and **4** (50 mg and 30 mg, respectively).

Compound 1: White needle-like crystals $C_{30}H_{50}O$, (100 mg), R_f 0.83, mp 216-218 ºC (lit. 214-215 ºC (Fotie et al., 2006; Lutta et al., 2008; Abdullahi et al., 2013).

^{1}H (CDCl3, 300 MHz, ppm): d_H 3.20 (H-3, 1H, dd, *J*=11.0, 4.8 Hz), 0.76 (H-23, 3H, s), 0.79 (H-24, 3H, s), 0.83 (H-25, 3H, s), 0.94 (H-26, 3H, s), 1.02 (H-27, 3H, s), 0.76 (H-28, 3H, s), 4.57 (H_a-29, 1H, d, *J*=0.4), 4.67 (H_b-29 1H, dq, *J*=0.5,0.5).

^{13}C NMR (CDCl3, 75 MHz, ppm): δ_C 38.7 (C-1), 27.2 (C-2), 77.7 (C-3), 38.7 (C-4), 54.0 (C-5), 18.6 (C-6), 43.3 (C-7), 41.7 (C-8), 49.2 (C-9), 37.5 (C-10), 21.3 (C-11), 37.9 (C-12), 38.0 (C-13), 41.6 (C-14), 28.6 (C-15), 35.9 (C-16), 42.9 (C-17), 49.2 (C-18), 48.0 (C-19), 149.7 (C-20), 30.0 (C-21), 39.6 (C-22), 27.3 (C-23), 14.9 (C-24), 16.0 (C-25), 15.8 (C-26), 14.5 (C-27), 17.4 (C-28), 108.7 (C-29), 19.9 (C-30). **EI-MS (rel. int):** *m/z* 426, 411 (M^+-Me), 385 (M^+-41), 355 (M^+-Me $3H_2O$), 220 (M^+-$C_{15}H_{26}$), 218 (M^+-$C_{14}H_{24}O$), 207 (M^+-$C_{16}H_{27}$), 189 (M^+-$C_{16}H_{29}O$). **IR ν_{max} (KBr) cm^{-1}:** 3315, 2900, 1650, 1462, 1190, 1037, 997, 681.

Compound 2: White crystals, $C_{30}H_{50}O$ (50 mg), R_f 0.62, mp 254-256 ºC [lit. 252-254 ºC (Majidul et al., 2015)].

^{1}H ($CDCl_3$, 300 MHz, ppm): δ_H1.76 (H-1, 2H, dd, *J*=13 and 7.5 Hz), 2.25 (H_b-2, d, *J*=6.8), 2.27 (Ha-2, 2H, dd, *J*=13, 7.5 Hz), 2.25 (H-4, 1H, m), 1.62 (H-6, 2H, dd *J*=11.4, 5.2Hz), 0.89 (H-23, 3H, d, *J*=7.5 Hz), 0.74 (H-24, 3H, s), 0.88 (H-25, 3H, s), 1.02 (H-26, 3H,s), 1.03 (H-27, 3H, s), 1.07 (H-28, 3H,s), 1.20 (H-29, 3H, s).

^{13}C NMR ($CDCl_3$, 75 MHz, ppm): δ_C 22.5 (C-1), 41.6 (C-2), 213.4 (C-3), 58.5 (C-4), 42.4 (C-5), 41.6 (C-6), 18.2 (C-7), 53.4 (C-8), 37.7 (C-9), 59.8 (C-10), 35.3 (C-11), 30.8 (C-12), 41.8 (C-13), 41.6 (C-14), 30.3 (C-15), 35.9 (C-16), 30.3 (C-17), 43.1 (C-18), 35.9 (C-19), 29.9 (C-20), 33.1 (C-21), 39.6 (C-22), 7.1 (C-23), 14.9 (C-24), 20.5 (C-25), 18.9

(C-26), 18.5 (C-27), 32.1 (C-28), 32.4 (C-29), 32.7 (C-30).

Compound 3: White needle-like crystals, $C_{29}H_{48}O$, (114 mg), R_f 0.48, mp 166-168 ºC (lit. 165-166 ºC (Alam et al., 1996; Reginatto et al., 2001; Orabi, 2011)

^{1}H (CDCl$_3$, 300 MHz, ppm): δ_H 1.64 (H-2, 2H, *J*=5.6), 3.52 (H-3, 1H, m), 2.19 (H-4, 2H, d *J*=9.8), 5.35 (H-6, 1H, d, *J*=5.2 Hz), 1.52 (H-7, 2H), 1.02 (H-18,3H, s), 0.78 (H-19, 3H, s), 5.19 (H-20, 1H, dd, *J*=14.8, 5.5 Hz), 4.98 (H-21, 1H, dd, *J*=15.2, 6.6 Hz), 1.25 (H-22, 1H, d, *J*=0.93), 1.10 (H-23, 1H, s), 0.68 (H-24, 3H, s), 0.78 (H-25, 1H,s), 0.78 (H-26, 3H, s), 0.78 (H-27, 3H, s), 1.25 (H-28,3H, s), 0.81 (H-29, 3H, s).

^{13}C NMR (CDCl$_3$, 75 MHz, ppm): δ_C 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 39.8 (C-4), 140.8 (C-5), 121.7 (C-6), 31.9 (C-7), 31.7 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 37.2 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.3 (C-16), 56.1 (C-17), 11.7 (C-18), 19.3 (C-19), 129.2 (C-20), 138.1 (C-21), 45.9 (C-22), 23.1 (C-23),11.9 (C-24), 29.2 (C-25), 19.0 (C-26), 19.6 (C-27), 19.4 (C-28), 12.0 (C-29). **ESI-MS (rel. int):** *m/z* 412, 367, 271, 255, 189, 175, 161, 133, 121, 105, 95, 81, 69, 41. **IR ν_{max} (KBr) cm^{-1}:** 3373, 2940, 2867, 1641, 1457, 1381, 1038.

Compound 4: Colourless needles, $C_{35}H_{58}O_6$, (65 mg), R_f 0.63, mp 290-292 ºC [lit. 289-290 ºC (Pandey et al., 2006; Alfian et al., 2012; Mahbuba et al., 2012)].

^{1}H (CDCl$_3$, 300 MHz, ppm): δ_H 5.13 (H-6, 1H, dd, *J*=4.7, 1.7 Hz,), 5.10 (H-22, 1H, dd, *J*=15.2, 8.8 Hz), 5.06 (H-25, 1H, dd, *J*=15.2 & 8.8 Hz), 4.95 (H-1', 1H, d, *J*=7.9 Hz), 4.87 (H-6'a, 1H, dd, *J*=11.7 & 2.4 Hz), 4.82 (H-6'b, 1H, dd, *J*=5.3, 11.7 Hz), 4.80 (H-3', 1H, *m*), 4.44 (H-4', 1H, *m*) 4.40 (H-2', 1H, *t*, *J*=7.9 & 8.8 Hz), 2.81 (H-5', 1H, *m*), 2.77 (H-7a, 1H, dd, *J*=12.3, 2.6 Hz), 2.37 (H-7b, 1H, dd, *J*=1.5, 11.3 Hz), 2.01 (H-8, 1H, *m*), 1.99 (H-9, 1H, *m*), 1.03 (Me-21, 3H, d, *J*=6.4 Hz), 0.96 (Me-19, 3H, *s*), 0.92 (Me-26, 3H, d, *J*=6.8 Hz), 0.90 (Me-27, 3H, d, *J*=6.8 Hz), 0.83 (Me-18, 3H, *s*), 0.88 (Me-29, 3H, *t*, *J*=7Hz).

^{13}C NMR (CDCl$_3$, 75 MHz, ppm): δ_C 37.2 (C-1) 28.9 (C-2), 78.1 (C-3), 41.2 (C-4), 140.8 (C-5), 121.9 (C-6), 31.1 (C-7), 31.8 (C-8), 50.2 (C-9), 36.5 (C-10), 20.2 (C-11), 39.9 (C-12), 42.3 (C-13), 56.4 (C-14), 23.5 (C-15), 28.4 (C-16), 56.4 (C-17), 11.8 (C-18), 19.3 (C-19), 35.8 (C-20), 18.7 (C-21), 137.5 (C-22), 127.3 (C-23), 45.5 (C-24), 27.3 (C-25), 19.2 (C-26), 18.7 (C-27), 25.9 (C-28),12.3 (C-29), 101.4 (C-1'), 74.0 (C-2'), 76.8 (C-3'), 70.5 (C-4'), 76.1 (C-5'), 62.1 (C-6'). **ESI-MS (rel. int):** *m/z* 574, 603.3 {M$^+$ (C_2H_5), (100), 601.3 (20), 583 (10). **IR ν_{max} (KBr) cm^{-1}:** 3392 (OH), 2931-2868 (aliphatic stretch, 1641 (C=C) stretch), 1432 (CH_2-stretch) 1369 (isopropyl stretch), 1256, 1164, 1061, 1017.

2.5. Analgesic effect in the hot plate test

The modified method of Eddy and Leimbach, 1953 was used. Groups of mice (5 per group) of either sex (17-30 g) were used as test organisms. The mice were initially screened by placing them in turn on a hot plate (Electrothermal Eng. Ltd) set at 55±1 ºC and animals which failed to lick the hind paw or jump within 15 s were discarded (nociceptive responses). Eligible animals were divided into five groups and the pre-treatment reaction time for each mouse was determined before drug treatment so that each animal served as its own control. The times until the animals licked the paw, flutter any of the paws or jump was taken as reaction time and were recorded with the aid of an inbuilt stopwatch. Mice in the different groups were then treated with 0.8% normal saline water which doubled as a vehicle [10 mL/kg, per oral (p.o)], the ethyl acetate extract of the leaves of *L. eriocalyx* (100 mg/kg, p.o), together with compounds **1**, **2**, **3** and **4** (10 mg/Kg) and morphine (10 mg/kg, s.c) as the positive control. The latency was recorded after 30 and 60 min following oral administration of extracts (100 mg/kg), normal saline (10 mL/kg) and subcutaneous administration of morphine (0.5 mg/kg). A post-treatment cut off time of 30 s was used to avoid paw tissue damage (Omisore et al., 2004).

%Inhibition=[Post-treatment Latency]−[Pre-treatment Latency]/[Cut-off Time−Pre-treatment Latency]×100 (**Eqn. 1**)

2.6. Acetic acid (chemical-induced) writhing method

Abdominal writhes consist of contraction of the abdominal muscle together with the stretching of the hind limbs, induced by intra-peritoneal injection (i.p) in mice of acetic acid (0.8% solution in normal saline, 0.1 mL/10 kg), the nociceptive agent (Koster et al., 1959). Ethyl acetate extract of leaves of *L. eriocalyx* (100 mg/kg, p.o) and compounds **1**, **2**, **3** and **4** (10 mg/kg, p.o) were administered to mice (animals fasted overnight and divided into five groups of six animals) 60 min before (i.p) of acetic acid (0.6%, v/v in normal saline, 10 mL/kg, i.p). Normal saline was used as the control. The number of writhes which was characterized by contraction of the abdominal musculature and extension of the hind limbs) was counted for 30 min at 5 min interval of intra-peritoneal injection of acetic acid (Adeyemi et al., 2004). Statistical analysis results obtained were expressed as mean±standard error of mean (SEM) or standard deviation (SD). The data were analyzed using one way ANOVA followed by Bonferroni posttests and Dunnett's multiple comparison tests. Values were considered significant when $p \leq 0.05$.

Inhibition(%)=Number of Writhes [Control]−Number of Writhes [Treatment]/Number of Writhes [Control]×100 (**Eqn. 2**)

3. Results and Discussion

Repeated column chromatography separation of the ethyl acetate extract of the leaves of *L. eriocalyx* (30 g) yielded four compounds (**1-4**): Fig. 1. compound **1**, white needle-like crystals; with a molecular formula $C_{30}H_{50}O$ evidenced by a molecular ion at *m/z* 426 [M$^+$] gave a positive stable violet ring with Libermann-Burchard test

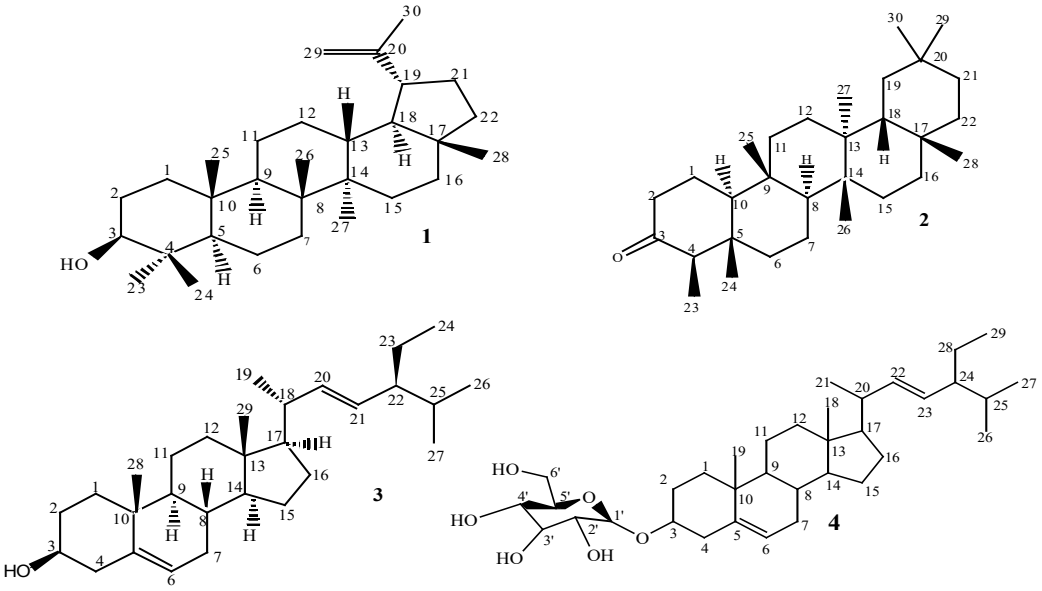

Fig. 1. Structures of the isolated and identified compounds: **1**, **2**, **3** and **4**.

indicating a triterpenoid or steroid skeleton (Attarde et al., 2010). The ^{1}H and ^{13}C NMR spectra had signals consistent with a pentacyclic lupane-type triterpene with olefinic protons of an exocyclic double bond at δ 4.57 and 4.67 (2H, m, Hα-29 and Hβ-29). The latter signals were confirmed by the appearance in the ^{13}C NMR of olefinic carbons at δ 149.7.0 for C-20; extremely downfield due to the electron donating effect of the methyl group at C-30 and another signal at δ 108.1 for C-29 further upfield. Both ^{1}H and ^{13}C NMR showed the signal typical of hydroxymethine proton (H-3) at δ 3.20 (dd, *J*=11.0, 4.8 Hz) and carbon (C-3) δ 77.7, respectively which was attributed to a proton germinal to an alcoholic group (Thanakijcharoenpath and Theanphong, 2007). Seven singlet signals of tertiary methyl protons at δ 0.76 (2 x CH_3), 0.79, 0.83, 0.94 and 1.02, 1.20 (integrating for each 3H) with corresponding ^{13}C NMR signals at 27.3, 14.9, 16.0, 15.9, 15.0, 18.0 and 19.5, respectively as deduced from the HMQC which were in agreement with the structure of lupeol. The confirmation of this structure was accomplished through extensive analysis of the 2D NMR experiments of COSY, HMBC and HMQC (Abdullahi et al., 2013).

Comparison of ^{1}H NMR and ^{13}C NMR spectra of compound **1** with those of lupeol previously isolated from *Lonchocarpus sericeus*, *Holarrhena floribunda* were all in agreement with the structure of lupeol (Fotie et al., 2006; Lutta et al., 2008; Correa et al., 2009). IR ν_{max} (KBr) cm^{-1}: 3315, 2900, 1650, 1462, 1190, 1037, 997, 681 which were typical of the functional groups in lupeol. The identity of this compound as lupeol has been confirmed by Co-TLC with authentic specimen (Abdullahi et al., 2013).

Compound **2** (50 mg) was obtained as white crystals mp; 254-256 °C with a molecular formula of $C_{30}H_{50}O$. The ^{1}H, ^{13}C NMR had a signal for H-1a at δ 1.76 and H-2b at δ 2.27 (dd) integrating for 1 proton each. A signal for H-4 was observed as a multiplet integrating for one proton at δ 2.25, while H-6 was at δ 1.62 (dd) with corresponding C-2 and C-6 at δ 41.6 and 41.8, respectively (Majidul et al., 2015). Eight signals typical of methyl protons were also observed at δ 0.74, 0.88, 0.89, 0.91, 1.02,1.03 and 1.07, 1.20 with corresponding ^{13}C NMR signals at δ 32.4, 32.7 for Me-29 and Me-30, 7.1 for Me-23, and 18.9 for Me-26. Signals typical of methylene carbons were also observed at δ 58.5, 53.4 and 59.8 for C-4, C-8 and C-10, while quaternary carbon signals were observed at δ 29.9 and 30.0 for C-20 and C-17. Also, observed were eleven signals typical of methylene carbons at δ 18.2, 22.5, 29.9, 32.7, 33.05, 35.3, 35.6, 35.9, 36.3, 39.5, 41.6. In fact, the presence of signals due to one secondary and six quaternary methyls in the ^{13}C NMR spectrum together with cross correlation between a doublet at methyl signal at δ 1.20 and a quartet of methine at δ 2.27 in the ^{1}H-^{1}H COSY spectrum strongly supported the structure of the compound as friedelin (Grasiely et al., 2012; Majidul et al., 2015). Thus, on the basis of spectroscopic evidence, compound **2** was identified as friedelin.

The C=O carbon was confirmed by signal at 213.4 typical of a ketonic carbon (Majidul et al., 2015). Mass spectral fragmentation pattern was quiet typical of pentacyclic ring structure, containing seven methyl groups in rings other than A (*m/z* 341) (Majidul et al., 2015). The mass peak at *m/z* 273 indicated that the carbonyl oxygen is present in ring A, B or C. Another peak at *m/z* 302 was proof for the presence of three methyl groups in ring E. In the mass spectrum, peak at *m/z* 341 originated by the loss of ring A, the peak at *m/z* 302 by the loss of ring E, and the peak at *m/z* 273 by the loss of ring D and E (Majidul et al., 2015).

Compound **3** was isolated as white needle-like

Table 1

Effects of crude extracts and pure compounds of *Lonchocarpus eriocalyx* on hot plate-induced pain and acetic acid-induced writhing in mice.

Dose Treatment	Pretreatment latency (s)	Pain threshold (time lapse after treatment)									Writhing response	
		Post treatment latency (s) and % inhibitions										
	0 min	30 min		60 min		90 min		120 min			Total no. of writhes	% Inhibition
	sec	sec	% Inb	sec	% Inb	sec	% Inb	sec	% Inb			
Vehicle (0.8% normal saline)	3.4±0.15	3.4±0.15	NS	5.2±0.14	6.76	5.8±0.20	9.02	5.8±0.15	9.02		86.3±4.04	0.00
n-Hexane	3.9±0.01	5.0±0.1	4.51	30.5±0.20	40.50	11.2±0.20	26.51	6.9±0.20	13.32		23.7±2.54	37.69
EtOAc	3.1±0.15	**6.4±0.13**	6.04	12.3±0.31	35.04	11.0±0.25	22.92	**8.5±0.20**	14.33		24.3±1.49	**50.52**
DCM	3.0±0.01	4.4±0.04	**8.23**	11.5±0.25	32.16	7.4±0.20	25.13	6.1±0.21	13.55		27.3±2.51	36.05
MeOH	3.5±0.12	4.8±0.10	7.33	12.0±0.31	33.18	12.6±0.20	28.52	6.9±0.25	11.98		34.7±2.63	53.70
Cpd **1**	3.1±0.12	**6.8±0.10**	**10.33**	**17.0±0.31**	**53.18**	**20.6±0.20**	**56.52**	**9.5±0.25**	**30.98**		**14.7±2.63**	**76.70**
Cpd **2**	3.2±0.12	**6.8±0.13**	**7.97**	**15.1±015**	38.09	16.4±0.10	41.94	7.5±0.13	**38.31**		**19.7±2.08**	**66.47**
Cpd **3**	3.2±0.15	4.8±0.10	5.29	8.0±0.10	13.5	5.8±0.22	8.51	7.3±0.23	**31.90**		**21.3±2.50**	**62.24**
Cpd **4**	3.6±0.11	4.1±0.14	6.97	8.1±015	27.31	8.4±0.12	19.94	7.5±0.13	22.61		35.3±4.53	45.44
Morphine (0.5 mg/Kg)	2.9±0.15	7.2±0.15	15.86	20.4±0.20	64.57	23.3±0.57	75.27	15.8±0.35	47.6		NT	
Acetyl salicyclic acid											**10.9±1.10**	**87.37**

Values are mean ± SEM (n=6); NS=Non significant ($p < 0.05$) vs. control (one-way ANOVA followed by Bonferroni posttests); NT=Not tested.

crystals and the ESI-MS indicated a molecular ion peak at *m/z* 412 suggesting a molecular formula of $C_{29}H_{48}O$, (114 mg), mp; 166-168 ºC. The ^{1}H and ^{13}C NMR spectra had a multiplet signal for H-3 at δ 3.52 for the oxymethine proton which suggested the presence of an α-proton typical of sterols hydroxylated at C-3 (Orabi, 2011). A δ_C signal at 71.8 in the oxygenated aliphatic region confirmed the presence of the oxymethine carbon (Orabi, 2011). A signal for H-6 typical of olefinic proton of appeared at δ 5.35 (d, *J*=5.2 Hz), while two other olefinic protons appeared upfield at δ 5.01 (dd, *J*=15.2, 6.6 Hz) and 5.40 (dd *J*=12, 6.0 Hz) which were confirmed by carbon-carbon double bond resonances at δ_C signals at 129.2 (C-20) for a disubstituted carbon and 138.1 (C-21), respectively; and this confirmed the presence of two double bonds. Another olefinic carbon signal appeared downfield at 140.8 (C-5) typical of a trisubstitution compared to a δ_C signals at 121.7 for C-6. The presence of six methyl protons was confirmed by signals at 0.69, 0.78 (2 x CH_3), 0.93, 1.02 and 1.25 with corresponding ^{13}C NMR signals at δ 19.3, 11.9, 19.4, 19.6, 19.0 and 12.0, respectively (Alam et al., 1996; Reginatto et al., 2001). The appearance of six methyl signals suggested the presence of a sterol. ^{13}C NMR spectra showed the presence of 29 carbon atoms which included six methyls, nine methylenes, eleven methines and three quaternary carbons in total. This compound was identified as stigmasterol based on spectral data as well as comparison with information contained in literature (Alam et al., 1996; Orabi, 2001; Reginatto et al., 2001; Orabi, 2011).

Compound **4** was obtained as colorless needles (65 mg) with a molecular formula of $C_{35}H_{58}O_6$, mp; 290-292 ºC which was consistent with *m/z* of 574. Its ^{1}H

NMR spectrum showed olefinic proton H-6 at δ 5.13 ppm (dd, *J*=4.7, 1.7 Hz) as a double doublet because the two equivalent adjacent protons at (H-7) due to its close proximity of 19-Me group. Another set of olefinic protons resonated as two doublets of doublet at δ 5.10 (H-22, dd, *J*=15.2, 8.8 Hz) and δ 5.06 (H-23, dd, *J*=15.2, 8.8 Hz) which represented *trans* olefinic protons plus adjacent methine proton (Silverstein et al., 1991). The protons of its sugar moiety resonated at δ 2.81-4.95 ppm. This compound almost completely corresponded to the data for stigmasterol with the exception of the signals between H δ 2.81-4.95 ppm typical for a sugar moiety (Silverstein et al., 1991). The ^{13}C NMR spectrum of compound **4** revealed 35 carbon signals in the molecule. The olefinic carbon resonances at δ 121.9 (C-6), 137.5 (C-22), and 127.3 (C-23) were observed for the methine carbons, as well as a signal at δ 140.8 represented the C-5 quaternary carbon of the sterol moiety. A signal typical of an anomeric carbon at δ 101.4 (C-30) indicated the presence of a single monosaccharide moiety. Four other sugarcarbons resonated at δ 74.0 (C-2′), 76.75, 76.8 (C-3′, 70.5 (C-4′) and 76.1 (C-5′) as well as the methylene resonance at δ 62.1 (C-6′), respectively of the β-D-glucopyranoside (Ahmad et al., 2012). The presence of anomeric proton (H-30) was evident by a signal at δ 4.95 with diagnostic *J*-value of 7.9 Hz (H-30, 1H, d, *J*=7.9 Hz) and this reflected that the proton is the axial-axial to H-31 which means glucopyranoside moiety binds to the sterol moiety at β-position (Silverstein et al., 1991). Extensive interpretation of the spectral data coupled with comparison with spectroscopic data contained in literature led to unequivocal identity of the structure of compound **4** (Ahmad et al., 2012). The relationship in the bonding structure was proven through long-range correlation of ^{1}H and ^{13}C of HMBC spectrum. The existence of long-range correlations of protons at δ 4.95 (H-1′) with a carbon at δ 78.1 (C-3) and 76.1 (C-5′) indicates that the group of glucose is bound to C-3 (oxy carbon sp3) (Pandey et al., 2006; Alfian et al., 2012; Mahbuba et al., 2012). Conclusive evidence for the structure of compound **4** was further provided by the extensive interpretation of ^{1}H and ^{13}C chemical shift correlations experiment which further supported both ^{1}H and ^{13}C NMR results (Ahmad et al., 2012). Therefore, on the basis of the above accumulated evidence, the structure of **107** was established as stigmasterol 3-*O*-β-glucoside.

3.1. Analgesic effect in the hot plate test

The analgesic effect of the crude extracts from the leaves of *Lonchocarpus eriocalyx* and the isolated compounds were studied in mice using hot plate-induced pain. Preliminary results showed that the pretreatment latency for morphine (2.9±0.15 s) was quite comparable to that of crude extract (100 mg/Kg) at the zero minute whose values were 3.1±0.15 and 3.0±0.01 for EtOAc and DCM, respectively implying that they delayed infliction of pain more/less with the same magnitude as the standard drug just as the instant time of administration. Similarly, the EtOAc extract had a significant effect in delaying the pain within 30 minutes which was quite comparable to that of morphine (10 mg/Kg) meaning longer post treatment latency. Generally, the crude extracts significantly increased the reaction time for nociception from the beginning to 60 minutes post treatment. However, the effects of the crude extracts (100 mg/kg) were significantly ($p<0.05$) lower than those produced by morphine in the same tests. Lupeol (**1**) and friedelin (**2**) delayed incubation of pain from the beginning to 60 minutes after which the effect was insignificant. Lupeol and friedelin had longer latency compared to the crude extracts from the beginning to 60 minutes suggesting that purity enhanced the efficacy of the compounds. Both the extracts and isolates exhibited significant analgesic effect as shown in Table 1.

3.2. Acetic acid (chemical-induced) writhing method

Acetic acid-induced writhing test in mice was also used to study the analgesic effect of the crude extracts and the isolates. After intraperitoneal injection with the crude extracts of the leaves of *L. eriocalyx* comparatively less number of writhes was observed (contraction of abdominal muscles together with stretching of the hind limbs) implying that the extracts had significant ability to relieve pain. A percent inhibition of 50.52, 76.7, 66.47 and 62.24% was observed in ethyl acetate for the compounds **1**, **2** and **3**, respectively.

Similarly, the total number of writhes of compounds 1, 2 and 3 observed were 14.7±2.63, 19.7±2.08 and 21.3±2.50, respectively. The obtained results are summarized in Table 1.

4. Concluding remarks

This study led to the isolation of four natural compounds, of which two were reported for the first time in the literature. The results obtained confirm the folkloric information contained in literature that this plant (*L. eriocalyx*) has analgesic activity and is also used in managing fever. In conclusion, our study revealed that this herbal plant could be successfully used as an herbal remedy.

Conflict of interest

The authors declare that there is no conflict of interest.

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