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Isolation and analgesic property of lupeol from *Diospyros mespiliformis* stem bark

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Diospyros mespiliformis **Hochst (*Ebenaceae*) stem bark is used in traditional medicine for the management of pain related ailments. Several bioactive compounds have previously been isolated from the plant material that includes pentacyclic triterpenes. This study sequentially extracted and carried out a bioassay-guided fractionation of the plant crude material with solvents of varying polarity using analgesic efficacy in rats as bioactivity marker, aimed to isolate the active constituent. Powdered stem bark of the plant was sequentially extracted with hexane, chloroform and methanol; and preliminary tested for analgesic activity. The chloroform extract being the most active amongst the three extracts was subjected to column chromatography, and a fraction was eluted with mixture of hexane and ethyl acetate (50:50%) which yielded a compound. Three dose levels (25, 50 and 100 mg/kg) of the compound were administered orally to rats. Acetylsalicylic acid (100 mg/kg, p.o.) was used as the positive control. Nociception was induced mechanically using analgesy meter, and chemically with formalin. The compound alleviated the pain stimulus induced by the analgesy-meter and formalin in rats. The isolated compound was identified as lupeol using thermo-analysis (DSC), colorimetric, chromatographic and spectrometric techniques that included: UV-visible, IR, and ^{13}C- and ^{1}H NMR. It was concluded that lupeol acting alone or synergistically might be responsible for the beneficial effect of the plant in treatment of pain related ailments**.

Key words: *Diospyros mespiliformis, lupeol,* analgesic.

INTRODUCTION

Diospyros mespiliformis Hochst. ex A.DC. -- Prodr. (A. P. de Candolle) 8: 672. 1844 (mid Mar 1844) (IK) family: Ebenaceae (http://www.ipni.org), is a tree with white fragrant flowers and soft sweet pulp fruit that grows wild in tropical regions of Africa and Asia. The plant is reputed for its medicinal values, and is used in ethnomedical

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practice for treating various ailments that include sleeping sickness, malaria, headache, cough, leprosy, helminth infection (Belemtougri et al., 2006) and toothache (Etkin, 1981). Its seeds are also known to have nutraceutical value in managing high cholesterol, reducing risk of type-2 diabetes, and for weight control (Chivandi and Erlwanger, 2011). Useful biologically active compounds including naphthoquinone epoxide, α-amyrin, β-sitosterol, betulin and betulinic acid amongst others were isolated from the plant (Lajubutu et al., 1995; Mohamed et al., 2009).

Despite some advantages in the medical use of plant extracts over isolated entities, there is a need to identify the component which is responsible for the observed beneficial effects. This study sequentially extracted and fractionated the stem bark of *D. mespiliformis* in a bioassay-guided manner using analgesic activity in rats as bioactivity marker in order to identify its active component.

MATERIALS AND METHODS

Plant material

D. mespiliformis was collected at Chaza village, near Suleja (9°10'49 N; 7°10'45 E), Niger State, Nigeria. It was authenticated by Ibrahim Muazzam of Taxonomy Unit, Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. A voucher specimen (#5120) was deposited at the herbarium of the Institute*. D. mespiliformis* is not an endangered plant species, and therefore its collection for purposes of use and scientific study does not require prior authorization.

Extraction, fractionation and compound isolation

Stem bark of the plant was collected, cleaned, dried under shade and grinded into powder. The powdered material (500 g) was sequentially extracted with hexane (DM-1), chloroform (DM-2) and methanol (DM-3) to yield 0.97, 1.23 and 7.16% of the extracts, respectively. The three extracts (DM-1, 2, 3) were preliminary tested for analgesic potency in a pilot experiment (data not shown) using the formalin test (described below); being a model in which both peripheral and centrally mediated pain relief could be measured. The chloroform extract (DM-2) was found to be the most active amongst DM-1, 2, and 3; and was subjected to column chromatography (Still et al., 1978) using silica gel 230 to 400 mesh (Sigma-Aldrich Co., St. Louis, MO, USA). The column was eluted first with hexane, followed by mixtures (100 ml) of hexane: ethyl acetate; and ethyl acetate: methanol in increasing polarity gradient. Fifty ml of the eluates were individually collected, monitored with analytical TLC on precoated silica gel adsorption plates with 250 micron layer thickness (Whatman K5 150 A, Waltham, MA, USA) and visualized under Ultraviolet (UV) light (254/365 Eagle Scientific Ltd, UK). Eluates which were found to have the same thin-layer chromatography (TLC) profile were combined together. A fraction (initially denoted DM-2B) was eluted with mixture of hexane and ethyl acetate (50:50%), and on drying yielded a compound.

Structural elucidation

The isolated compound was identified using calorimetric, colorimetric, chromatographic and spectroscopic techniques. Differential scanning calorimeter (DSC) (NETZSCH DSC 204F1, Netzsch-Gerätebau GmbH, Selb, Germany) was used for thermochemical analysis. UV-visible spectra were recorded on UV-160A instrument (Shimadzu Corporation, Kyoto, Japan) by recording the absorption of 1 mg of the isolated compound in 10 ml ethanol (99%). IR spectra were taken in KBr pellets (FTIR-8400 S (CE), Shimadzu, Japan). The ^{13}C– and ^{1}H NMR spectra including 2-dimensional ^{1}H–^{13}C and ^{1}H–^{1}H correlation spectroscopy (COSY) were recorded on Bruker DRX 500 NMR (Bruker BioSpin, Rheinstetten, Germany) equipped with 5-mm QNP probe, 2H lock switch box and BVT 2000 heater. $CDCl_3$ was used as solvent and Tetramethylsilane (TMS) as internal reference. The chemical shifts were recorded in δ (ppm) and coupling constant in Hz. Distortion enhancement by polarisation transfer (DEPT) analysis was performed for proton attachment, heteronuclear multiple bond correlation (HMBC) for proton-carbon (^{1}H–^{13}C) ^{1}H–^{1}H coupling and correlation spectroscopy (COSY) coupling constants.

Animals

Wistar rats of both sexes obtained from Animal Facility Centre, NIPRD, Abuja, Nigeria, were used for the study. The animals were kept in propylene cages with saw-dust as bedding, and maintained on standard laboratory feeds with water ad libitum. They were used in accordance with Ethical Guidelines for Investigation of Experimental Pain in Conscious Animal (Zimmermann, 1983), in line with NIPRD's standard procedures on laboratory animal usage (NIPRD QMS/SOP no. 05:3:06).

Analgesy (Randall-Selitto test)

This test was performed using the modified Randall-Selitto (1957) test with Ugo Basile Analgesy-Meter (No. 7200, Italy). In the test, a meter exerts force at a constantly increased rate on rat paw monitored by a pointer moving along a linear scale. Twenty five rats were grouped into five groups (n = 5) and treated p.o. with vehicle (distilled water; 10 mL/kg), lupeol (25, 50 and 100 mg/kg), or acetylsalicylic acid (ASA) (100 mg/kg). The rat paw was gently placed between the plinth and plunger of the instrument and increased pressure (exerted by 20 g) applied to the middle dorsum of the rat's left hind paw. Stimulus was terminated and force threshold readings taken as soon as nociceptive response were elicited by the rats. Readings were taken pretreatment and at 15, 30 and 60 min after treatment.

Formalin test

The method described by Dubuission and Dennis (1977) was adopted for this assay, with little modification (Adzu et al., 2014). Briefly, the animals were treated p.o. with water (10 mL/kg), lupeol (25, 50 and 100 mg/kg), or acetylsalicylic acid (ASA) (100 mg/kg). They were then injected s.c. with 50 µL solution 2.5% formalin into the sub-plantar surface of rat left hind paw, 30 min after the treatment. Severity of pain was rated in two distinct phases for 60 min: the first phase (0 to 10 min) taken every 2 min and late phase (15 to 60 min) every 5 min using 3 pain-induced behaviour in the following scoring manner: 0 - normal weight baring on the injected paw; 1 - light resting on the paw on the floor; 2- elevation of the injected paw and 3 - for licking, biting or grooming of the injected paw. The mean (±SEM) of the readings was recorded as the pain score, after which the left paw oedema volume of each rat was measured and compared with that of the right hind paw using a digital plethysmometer (LE 7500, LETICA, Spain) 1 h after the formalin injection.

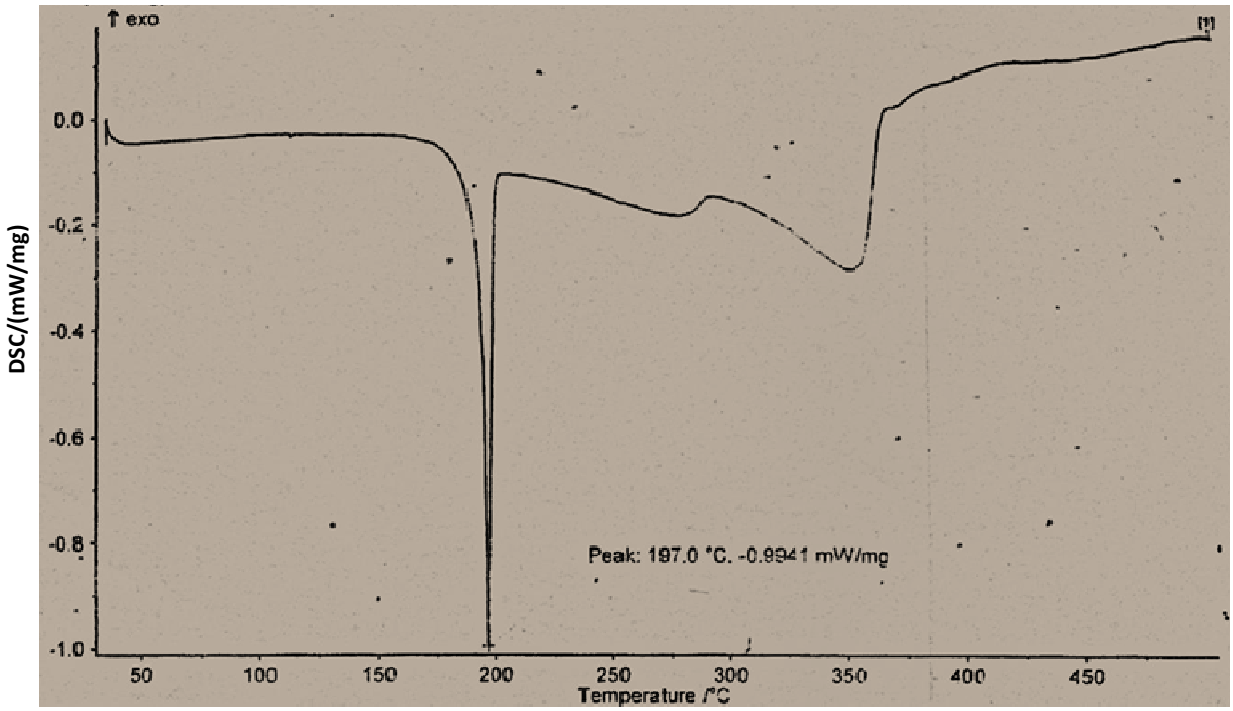

Figure 1. DSC spectra of lupeol isolated from the $CHCl_3$ extract of *Diospyros mespiliformis* stem bark

Data analysis

The results were expressed as mean ± standard error of mean. Parametric one way-analysis of variance (ANOVA) was used to analyse the data followed by the Student-Newman-Keuls test for multiple comparison using GraphPad Prism Version 5.01 for Windows (GraphPad Prism Software, San Diego California, USA).

RESULTS AND DISCUSSION

In a previous study, crude extracts of *D. mespiliformis's* pain and fever relief activity in rodents was shown (Adzu et al., 2002). This study carried out a bioassay-guided investigation to identify the active compound using analgesic effect in rats. In the course of the evaluation, a fraction eluted by mixture of hexane/ethyl acetate (50:50%) from the $CHCl_3$ extract was obtained. On drying, it yielded a white powdered compound. Calorimetric, colorimetric, chromatographic and spectroscopic investigations of the compound showed: mp 197°C on DSC (Figure 1); UV $_{MeOH}$209 nm; and IR 771.55 (C–H), 1043.52 (C–O), 1215.19 (CH_3–C), 2926.11 (C–H) and 3018.70 cm^{-1} (O–H). The NMR spectra signals were obtained for: ^1H (Fig. 2); ^{13}C (Figure 3); and ^1H–^1H COSY (Figure 4). The highlights are: one H protons (δ 4.69 and 4.59 ppm), and carbons (δ 109.32 and 150.98 ppm); hydromethine proton (δ 3.19 ppm) and carbon (δ 79.05 ppm); singlet signals (δ 0.77, 0.80, 0.84, 0.95, 0.98, and 1.04) assigned to tertiary methyl group; and absence of aromatic proton (δ 6 to 8 ppm). Other details were shown in Figures 2 to 4. The assignment of these NMR signals, aided by the UV-visible, IR and DSC data; and comparisons with relevant literatures (Igoli and Alexander, 2008; Bagalkotkar et al., 2011) identified the compound as lup-20(29)-en-3β-ol (lupeol; Figure 5).

The analgesic potency of the isolated compound was investigated using rats. Such animal models generate reliable data that gives high predictive value in humans (Normandin, 2007); by identifying target and provide proof of efficacy (Hart et al., 2004). In some instances, these in vivo models have advantages over vitro techniques (Houghton et al., 2007). The compound was first tested on mechanical model using analgesy-meter. The test is based on the principle that inflammation increases the sensitivity to nociception and this sensitivity is susceptible to modification by analgesics. The average pre-treatment response of the rats to the model was 4 min, which was maintained throughout the 60 min duration of the experiment by the vehicle control groups. Lupeol and the standard drug (ASA) alleviated the induced pain by prolonging the rats' responses significantly ($p< 0.05$; Table 1). The fact that lupeol increased the threshold of the intact paw suggests analgesic effect involvement of both peripheral and centrally mediated activity (Vongtau et al., 2004).

The compound was also evaluated against chemically induced pain using formalin test. The test is biphasic, and measures pain of both neurological (first phase) and inflammatory origin (second phase). The test is recommended as a basic pain research for studying the

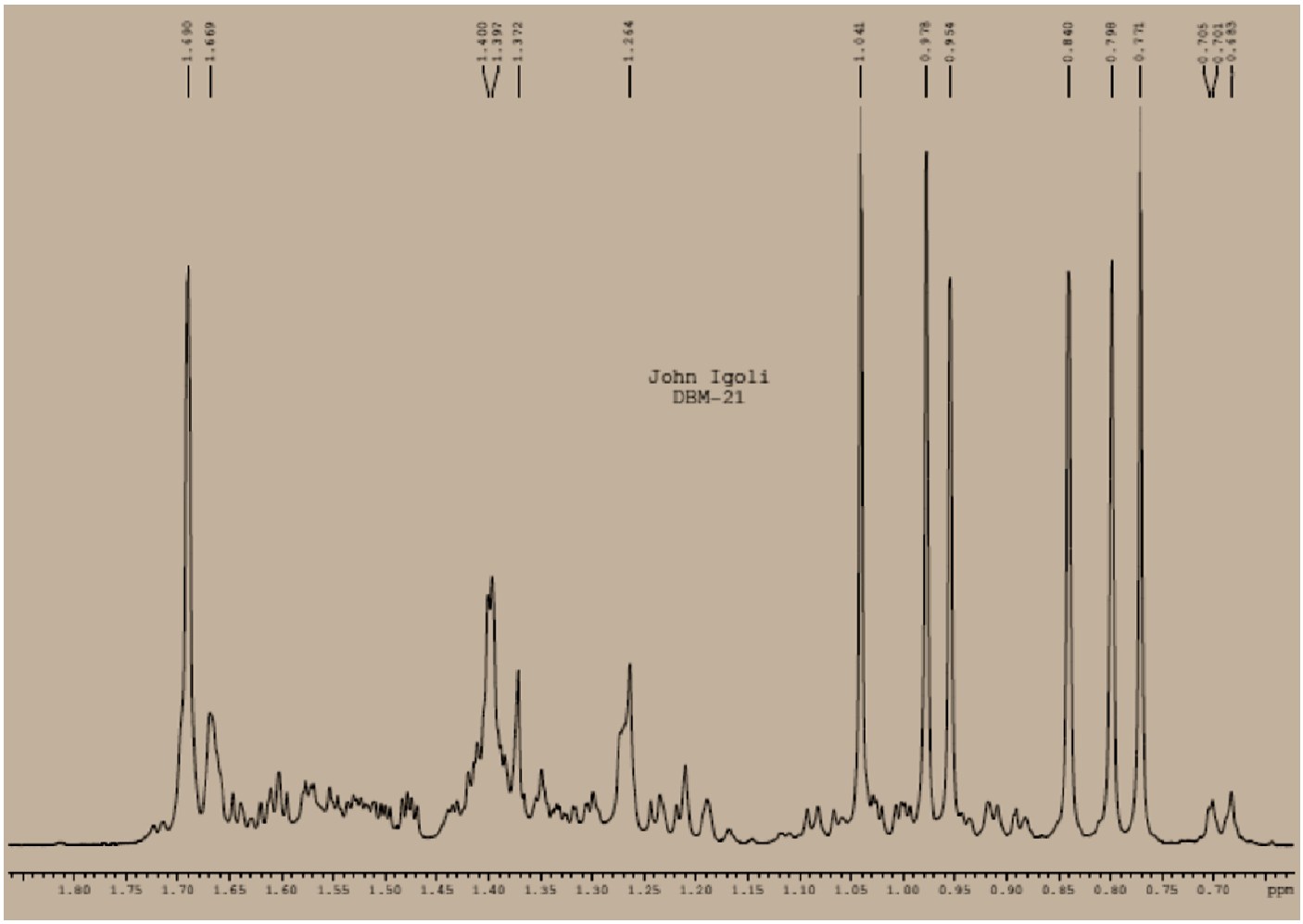

Figure 2. The ^{1}H NMR spectra of lupeol isolated from the $CHCl_3$ extract of *Diospyros mespiliformis* stem bark.

Table 1. Inhibition of analgesy in rats by lupeol isolated from the $CHCl_3$ extract of *D. mespiliformis* stem bark.

Treatment	Dose (mg/kg,p.o.)	Threshold (mean ± SEM)[a] at time (min)			
		Pre-[b]	15	30	60
Vehicle	-	4.16 ± 1.5	4.04 ± 1.1	3.80 ± 0.9	3.95 ± 0.9
Lupeol	25	4.07 ± 1.2	4.21 ± 0.9	5.06 ± 0.9*	5.72 ± 2.1*
	50	3.96 ± 0.4	5.62 ± 1.3*	6.16 ± 1.6*	6.38 ± 1.0*
ASA	100	4.60 ± 0.5	6.64 ± 1.8*	6.73 ± 1.0*	6.04 ± 2.2*
	100	4.24 ± 0.8	7.65 ± 0.9*	8.60 ± 1.7*	9.76 ± 1.7**

[a]Weight (20 g), mean ± SEM;[b] Pre-treatment; *n* = 6; one-way ANOVA, followed by Student-Newman–Keuls test for multiple comparison. *p< 0.05; **p < 0.01 vs. vehicle, ASA – acetylsalicylic acid.

mechanisms of analgesic agents because of its connection to tissue injury (Tjolsen et al., 1992). Lupeol exhibited significant analgesic activity on both phases of the formalin test; with maximal % inhibition of 60% in the first phase, and 31% at the second phase (Table 2). The formalin test model is accompanied by the development

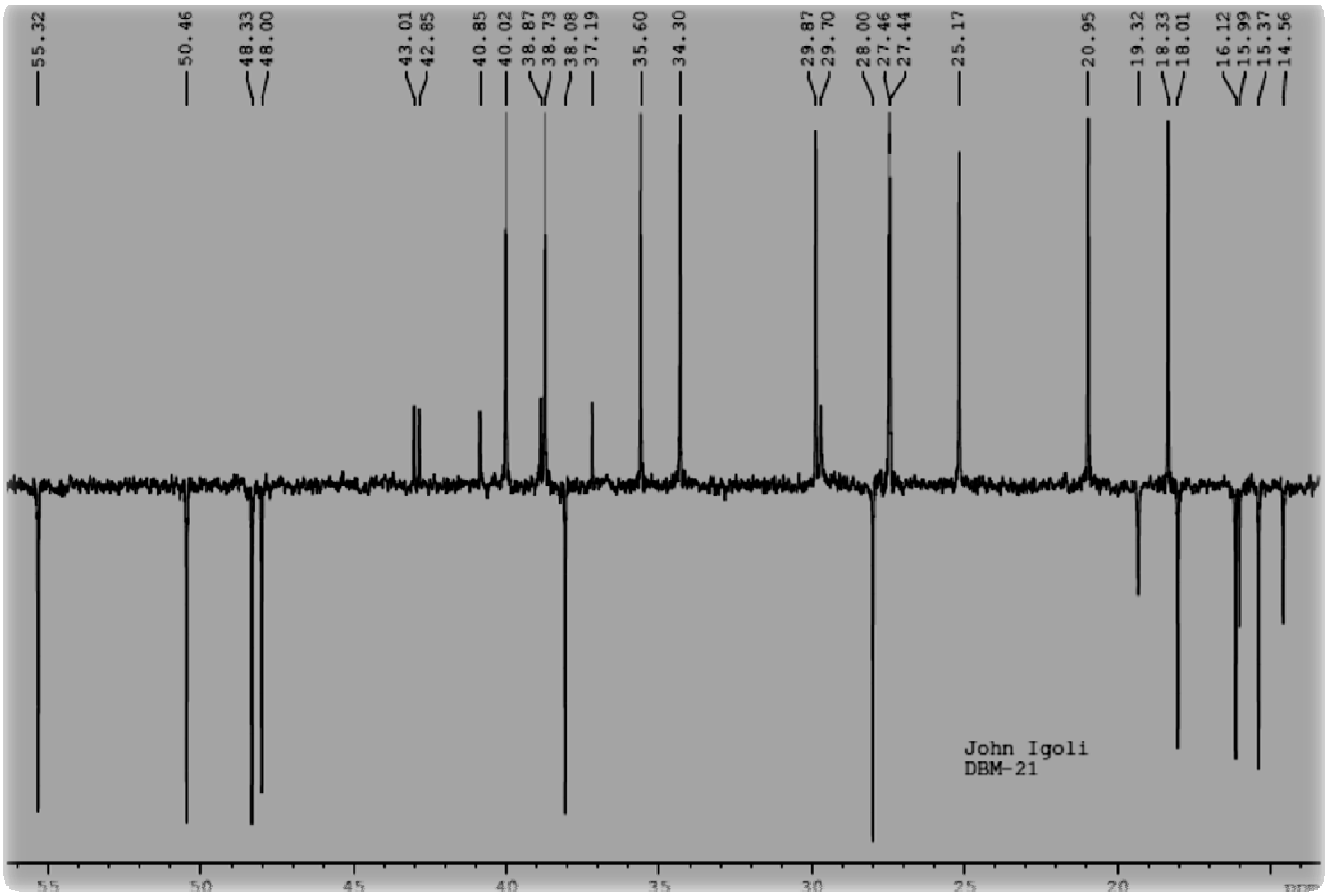

Figure 3. The ^{13}C NMR spectra of lupeol isolated from the $CHCl_3$ extract of *Diospyros mespiliformis* stem bark.

Table 2. Inhibition formalin induced noxious stimulus test in rats by lupeol isolated from the $CHCl_3$ extract of *Diospyros mespiliformis* stem bark.

Treatment	Dose (mg/kg, p.o.)	Pain inhibition (%)[a]		Oedema volume (cm^3)
		First phase	Second phase	
Control	-	2.56 ± 0.2	2.48 ± 0.1	0.37±0.04
Lupeol	25	2.2 ± 0.2*	1.98 ± 0.1	0.27 ± 0.02*
	50	1.75 ± 0.2*	1.72 ± 0.2*	0.24 ± 0.02*
	100	0.88 ± 0.2**	1.91 ± 0.1*	0.23 ± 0.02*
ASA	100	1.0 ± 0.3**	1.15 ± 0.1*	0.22 ± 0.04*

[a]Mean ± SEM, *n* = *6*; one-way ANOVA, followed by Student-Newman–Keuls test for multiple comparison. *$p < 0.05$; **$p < 0.01$ vs. vehicle; ASA – acetylsalicylic acid

of oedema in the injected left paw due to release of inflammatory mediators, and the oedema volume after the assay was taken to evaluate the action of lupeol against this process. Suppressing the induced pain and oedema by lupeol in this study reaffirmed its pain relief effect.

Lupeol, a safe and pharmacologically active triterpenoid is widely distributed in plant kingdom, but less applied in therapy (Gallo and Sarachine, 2009; Siddique and Saleem, 2011). It is known to elicit its activity mainly via the inhibition of tissue response to the induced nociception (Geetha and Varalakshmi, 2001; Chen et al., 2012), especially through the involvement of cytokines (De Lima et al., 2013).

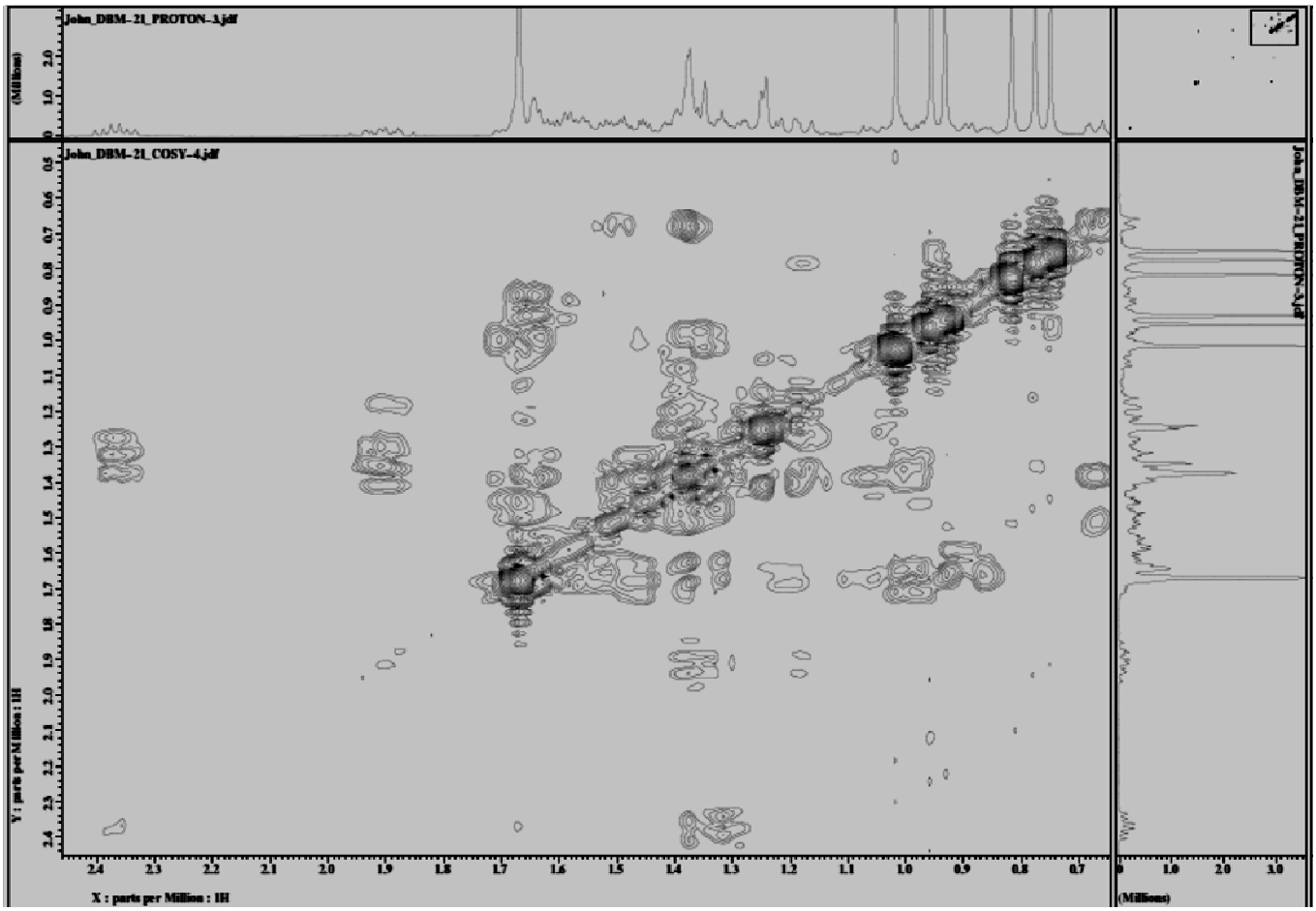

Figure 4. The ^{1}H –^{1}H COSY spectra of lupeol isolated from the $CHCl_3$ extract of *Diospyros mespiliformis* stem bark.

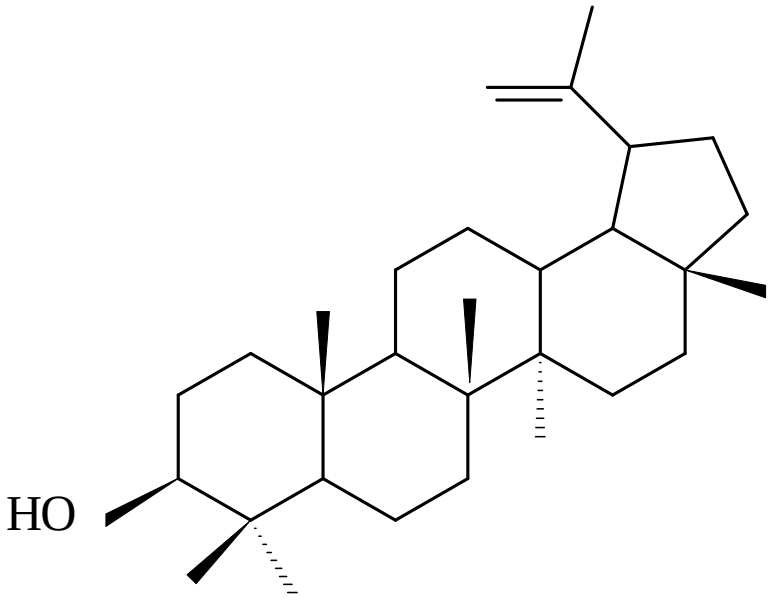

Figure 5. (lup-20(29)-en-3β-ol).

Conclusion

This is neither the first time lupeol is isolated from *D. mespiliformis* nor its bioactivity being demonstrated. However, this study is unique because it linked the analgesic effect of the plant material to the isolate lupeol; acting alone or synergistically with other phytochemical constituents. This might stimulate more interest in this compound since analgesics prototype like salicylic acid and morphine, and several other bioactive agents in modern pharmacopoeia were derived from products initially used in traditional medicine.

Conflict of Interest

The authors have not declared any conflict of interest.

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REFERENCES

Adzu B, Amos S, Dzarma S, Muazzam I, Gamaniel KS (2002). Pharmacological evidence favouring the folkloric use of *Diospyros mespiliformis* Hochst in the relief of pain and fever. J. Ethnopharmacol. 82:191-195.

Adzu B, Amizan MB, Okhale SE (2014). Evaluation of antinociceptive and anti-inflammatory activities of standardised rootbark extract of *Xeromphis nilotica*. J. Ethnopharmacol. 158:271-275.

Bagalkotkar G, Chuan JS, Khalivulla SI, Hamzah AS, Shaari K, Lajis NH, Saad MS, Stanslas J (2011). Isolation and cytotoxicity of triterpenes from the roots of *Phyllanthus pulcher* Wall Ex Műll Arg (Euphorbiaceae*)*. Afr. J. Pharm. Pharmacol. 5:183-188.

Belemtougri RG, Constantin B, Cognard C, Raymond G, Sawadogo L (2006). Effects of two medicinal plants *Psidium guajava*, L (Myrtaceae) and *Diospyros mespiliformis* L (Ebenaceae) leaf extracts on rat skeletal muscle in primary culture. J. Zhejiang Univ. Sci. B. 7:56-63.

Chen YF, Ching C, Wu TS, Wu CR, Hsieh WT, Tsai HY (2012). *Balanophora spicata* and Lupeol acetate possess antinociceptive and anti-Inflammatory activities *in vivo* and *in vitro*. Evid. Based Complement. Alternat. Med. 2012:371273.

Chivandi E, Erlwanger KH (2011). Potential usage of African Ebony (*Diospyros mespiliformis*) seeds in human health in: Nuts and Seeds in Health and Disease Prevention (Preedy VR, Watson RR, Patel VB Eds.), Elsevier B.V., Amsterdam, Netherlands. pp. 147-152.

De Lima FO, Alves V, Filho JMB, Da Silva Almeida JRG, Rodrigues LC, Soares MBP, Villarreal CF (2013). Antinociceptive effect of lupeol: Evidence for a role of cytokines inhibition. Phytother. Res. 27:1557-1563.

Dubuission D, Dennis SG (1977). The formalin test: a quantitative studyof the analgesic effect of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4:161-174.

Etkin NL (1981). A Hausa herbal pharmacopoeia: Biomedical evaluation of commonly used plant medicines. J. Ethnopharmacol. 4:75-98.

Gallo MBC, Sarachine MJ (2009). Biological activities of lupeol. Int. J. Biomed. Pharm. Sci. 3:46-66.

Geetha T, Varalakshmi P (2001). Anti-inflammatory activity of lupeol and lupeol linoleate in rats. J. Ethnopharmacol. 76:77-80.

Hart BA, Amor S, Jonker M (2004). Evaluating the validity of animal models for research into therapies for immune-based disorders. Drug Discov. Today 9:517-521.

Houghton PJ, Howes MJ, Lee CC, Steventon G (2007). Uses and abuses of in vitro tests in ethnopharmacology: Visualizing an elephant. J. Ethnopharmacol. 110:391-400.

Igoli OJ, Alexander GI (2008). Friedelanone and other triterpenoids from Hymenocardia acida. Int. J. Phy. Sci. 3:156-158.

Lajubutu BA, Pinney RJ, Roberts MF, Odelola HA, Oso BA (1995). Antibacterial activity of diosquinone and plumbagin from the root of *Diospyros mespiliformis* (Hostch) (Ebenaceae). Phytother. Res. 9:346-350.

Mohamed IE, El Nur EE, Choudhary MI, Khan SN (2009). Bioactive natural products from two Sudanese medicinal plants *Diospyros mespiliformis* and *Croton zambesicus*. Rec. Nat. Prod. 3:198-203.

Normandin S (2007). Claude Bernard and an introduction to the study of experimental medicine: "physical vitalism," dialectic, and epistemology. J. Hist. Med. Allied Sci. 62:495-528.

Randall LO, Selitto JJ (1957). A method for measurement of analgesic activity on inflamed tissue. Achiev. Int. Pharm. 111:409-419.

Siddique HRS, Saleem M (2011). Beneficial health effects of lupeol triterpenes: A review of preclinical studies. Life Sci. 88:285-293.

Still WC, Kahn M, Mitra A (1978). Rapid chromatographic technique for preparative separations with moderate resolution. J. Org. Chem. 43: 2923-2925.

Tjolsen A, Gerge O-G, Hunskaar S, Rosland JH, Hole K (1992). The formalin test: an evaluation of method. Pain 51:3-17.

Vongtau HO, Abbah J, Mosugu O, Chindo BA, Ngazal IE, Salawu OA, Kwanashie HO, Gamaniel KS (2004). Antinociceptive profile of the methanolic extract of *Neorautanania mitis* root in rats and mice. J. Ethnopharmacol. 92:317-324.

Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109-110.