FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 10 No. 4, February, 2026, pp 103 – 107
DOI: https://doi.org/10.33003/fjs-2026-1004-4743

FIRST ISOLATION OF LUPEOL FROM THE ETHYL ACETATE SOLUBLE FRACTION OF THE METHANOL ROOT BARK EXTRACT OF *Pterocarpus erinaceus* (POIR)

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ABSTRACT

Lupeol, a known pentacyclic triterpenoid was isolated from ethyl acetate soluble fraction of the methanol extract of the root-bark of *Pterocarpus erinaceus*. The plant is used in ethno-medicine to treat inflammation, hemorrhoid, cancer, wounds healing, worm expulsion and abdominal pain. One kilogram (1.0 kg) of the plant material was extracted with 2.5 L of methanol yielding 100 g (10.0 %) of the methanol extract using cold maceration method. The methanol extract was partioned with ethyl acetate and n- hexane yielding 20.5 g (2.05 %) and 15.6 g (1.56. %) respectively. Fifteen (15 mg) of 6.0 g (0.25 %) of the ethyl acetate soluble fraction was isolated by a combination of thin layer chromatography (TLC), column chromatography (CC) and preparative thin layer chromatography (PTLC). The structure of the compound which was coded as PEB2 was determined by analyses of its FTIR and MS spectral data as well as comparison with reported literature. FTIR showed broad absorption frequency at 3302.4 cm^{-1} which is typical of a hydroxyl group. A fairly intense bands at 2922.2 cm^{-1} and 2851.4 cm^{-1} that can be assigned to an aliphatic C-H (stretch) was observed. The C=C vibration frequency was observed at 1640.0 $cm^{`1}$. Mass spectral data gave a molecular formula $C_{30}H_{50}O$ with m/z 426. The compound appeared as white needle- like crystal with melting point of 217-218^{o}C. The compound was further confirmed by positive Liebermann - Buchard test for triterpenoid. This is the first report of isolation of Lupeol from the root bark of *Pterocarpus erinaceus*.

Keywords: Column chromatography, hemorrhoid, Isolation, Lupeol, *Pterocarpus erinaceus*

INTRODUCTION

Plant derived bioactive compounds have played an important role in the development of new or novel drugs. These compounds are synthesized by plants during their metabolic activities and are produced when the plant needs to adapt to certain changes within its environment. These compounds have very complex chemical structures and are known as secondary metabolites. Triterpenes are an example of a class of plant secondary metabolite. Research has proven that they play an important role in exerting various physiological actions in humans and animals (Howes, 2017). Lupeol is a pentacyclic triterpene reported to have important physiological and therapeutic effects in human health. In the past, studies have shown several important pharmacological activities of lupeol. In various *in-vitro* and *in-vivo* models, lupeol has been tested for its therapeutic efficiency against conditions such as inflammation, wound healing, arthritis, diabetes, cardiovascular diseases, renal disorder, hepatic toxicity, microbial infections and cancer (Tsai *et al.,* 2016). This phytosterol, is mostly found in fruits, and vegetables. It has been found to be pharmacologically effective in treating various diseases in preclinical settings (in animal models) regardless of the routes of administration such as, topical, oral, intra-peritoneal and intravenous. It has been reported to selectively target unhealthy human cells, while sparing normal and healthy once (Hifzur and Mohammad 2011). Because of it non-toxicity to normal cells and tissues, chemoprevention with it is a relatively new area in oncology (Palanimuthu *et al.,* 2012). *Pterocarpus erinaceus* Pori, commonly referred to as African rosewood or "Madobiya" in Hausa, is a multipurpose deciduous tree belonging to the Fabiaceae family. It is indigenous to the savannah and dry forest regions of West Africa, where it holds high ethnomedicinal relevance (Kouadio *et al.,* 2022). In traditional medicine, different parts of the plant such as the bark, leaves, and roots are used in the treatment of ailments including fever, diarrhea, ulcers, dysentery, malaria, respiratory infections, and skin diseases (Abubakar *et al.*, 2019). The tree typically grows to heights of 12–16 meters and is characterized by a fluted trunk, papery bark that peels off in flakes, and pinnately compound leaves. It produces yellow flowers and winged seed pods that are key to its propagation. (Orwa *et al.,* 2019). The medicinal uses of *P. erinaceus* includes the use of the leaves as a febrifuge, the bark is used for tooth and mouth troubles, the bark resin formed in to decoction is used as astringent for severe diarrhea and dysentery. The grated root bark is mixed with tobacco and smoked in a pipe as a remedy for cough. It has been found useful in the treatment of fever (Irvin, 2005). The decoction or infusions of the stem and root bark are used for treatment of tumors, urethral discharges, ring worm of the scalp, wounds and chronic ulcer. (Dalziel, 2007). The leaves, stem bark and roots of *P. erinaceus* are used in Burkina Faso to treat inflammatory diseases, ulcer, rheumatism, fever, bacterial infections, and malaria and stomach ache. Leaf decoctions are used to treat syphilis, fever and are also used as aphrodisiac. (Nadembega *et al.,* 2011). Member of the Fabaceae family are known to synthesize a wide range of secondary metabolites, including flavonoids, tannins, alkaloids, saponins, triterpenoids, and phenolic compounds (Akinmoladun *et al.*, 2020; Kouadio *et al.*, 2022). Flavonoids such as liquiritigenin, isoliquiritigenin, and pterostilbene derivatives have been isolated from some species of *Pterocarpus*. These compounds are known for their antioxidant, anti-inflammatory, and antimicrobial activities (Uba *et al.,* 2020). Studies using chromatographic and spectroscopic methods, including HPLC and NMR, have confirmed the presence of pterocarpans and stilbenes compounds typically linked to anti-cancer and antibacterial effects (Osei *et al.*, 2020; Arowosegbe and Olaleye, 2017). Alkaloids present in the plant extracts have also demonstrated broad-spectrum antimicrobial activity against bacterial and fungal pathogens. Similarly, tannins and saponins contribute to the plant's astringent and immunomodulatory properties, making it effective in wound healing and treatment of gastrointestinal disorders (Ajiboye *et al*., 2021). *Pterocarpus erinaceus*, traditionally used in African herbal medicine, has been shown to exhibit broad-spectrum antimicrobial activity. Methanolic stem bark extracts of this plant demonstrated significant inhibitory effects against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, and *Candida albicans* (Ajiboye *et al.,* 2021). Phytochemical studies on related species have reported the presence of bioactive steroids and triterpenoids, supporting the investigation of such compounds in *Pterocarpus erinaceus*. The antimicrobial potency is also largely attributed to the high content of triterpenoids, steroids, polyphenols, flavonoids, and other phytochemicals. In view of the above, this paper, reports the isolation of a pentacyclic triterpenoid Lupeol from the ethyl acetate soluble fraction of the methanol extract of the root bark of *Pterocarpus erinaceus*.

MATERIALS AND METHODS

Sample Collection, Identification and Extraction of the plant

The plant *P. erinaceus* was collected fresh from Taraba state, Nigeria in February 2023. It was authenticated by Dr Namadi Sunusi at the department of Biological science faculty of Life Sciences Ahmadu Bello University Zaria, Nigeria. A specimen voucher number 39185 was deposited at the Herbarium for reference purposes. *P. erinaceus* is not an endangered plant species, and therefore its collection for purposes of use and scientific study does not require prior authorization. The root bark was separated, air- dried and pulverized using a wooden mortar and pestle.

Extraction: The pulverized plant material (1 k**g**) was extracted using 2.5 L of methanol by cold maceration for 14 days to obtain hundred grams (100 g) yielding 10 % of the crude methanol extract. The methanol extract was then partitioned by suspending the hundred (100 g) in warm distilled water and filtered. It was partioned with ethyl acetate and n- hexane yielding 20.5 g (2.05 %) and 15.6 g (1.56. %) respectively (Dhawan and Gupta 2017). The ethyl acetate soluble fraction had previously been subjected to antimicrobial studies and found to exhibit good antimicrobial activity against selected pathogenic microbes (Uba *et al*., 2020; Gabriel and Onigbanjo, 2010).

Isolation Procedure and Column Chromatography

A small quantity of ethyl acetate soluble fraction was dissolved in chloroform and the solution was spotted on TLC plates. The plates were developed using several solvent systems; the solvent systems of Hexane / ethyl acetate (9:1) and hexane / Ethyl acetate (5:1) gave better separation of the components, and were used in the TLC monitoring of the column Chromatography. One hundred and twenty grams (120 g) of silica gel (60-120 mesh) was made into slurry with 100 % n-hexane and was packed into a 2.5 × 63 cm glass column and allowed to stand for about one hour to attain stability. 6.0 g of ethyl acetate soluble fraction was pre-adsorbed on 6.0 g of silica gel and loaded on to the column. The loaded sample was eluted gradiently starting with n-hexane (100 %), n-hexane: ethyl acetate (99: 1), n-hexane: ethyl acetate (98: 2), n-hexane: ethyl acetate (97: 3) and n-hexane: ethyl acetate (96: 4). A total of eighty (80) fractions of 100 ml each were collected and monitored by TLC. The spots on the TLC plate were visualized using ultraviolet light 254 nm as well as spraying with 10 % H_2SO_4 and placed in an oven already set at 105^O C for a few minutes to make the compounds more visible. The fractions were grouped into five namely PE1 (9 mg), PE2 (20 mg), PE3 (10 mg), PE4 (12 mg) and PE5 (10 mg) based on the similarity of their TLC profile. Fraction PE2 was found to have a significant proportion of the compound of interest and was further subjected to purification by preparative TLC. PTLC was carried out using Fluka silica gel precoated glass plates (20 x 20 cm) with layer thickness of 0.25 mm and using solvent system Hexane / Ethyl acetate (9:1). A single homogenous spot was obtained on TLC with two different solvent systems hexane / ethyl acetate (9:1) and (8:2). This compound (15 mg) coded PEB2 appeared as white needle- like compound was then subjected to spectral analysis.

Spectroscopic Characterization of the Isolated Compound PEB2

The structure of the compound was determined using Gas Chromatography-Mass Spectrometer GC-MS (Agilent 19091S-433UI, ABU Zaria - Nigeria with Fused capillary column (30× 0.25 m 1D × 0.25 m dF). Mass spectrometer operating in EI mode with ion source at 250°C and electron energy at 70 eV was used. Injector was set at 260° C with splitting ratio 1:10. One milligram (1 mg) of the isolated compound was dissolved in 200 μL chloroform (HPLC grade). The solution was filtered in order to remove any insoluble material which would block the sample introduction line Two (2 µL) of the sample was then injected into the mass spectrometer for analysis. The IR spectrum was recorded on FTIR-400s (Shimadzu) in CCl_4 at Ahmadu Bello University Multi User Laboratory. The compound (3 mg) was mixed with 5 mg of KBr and then ground to a very fine powder. The powder was compressed under high pressure using a press to produce pellets of the compounds to be analyzed. The pellets were then analysed.

Triterpenoid Test for Isolated Compound PEB2

The isolated compound (1 mg) was placed in a test tube, a few mg of Liebermann- Buchard reagent (glacial acetic acid + Conc.H_2SO_4) was added. The formation of red-violet color was observed at the interface of test tube indicates that the compound is a triterpenoid/ Steroids.

RESULTS AND DISCUSSION

Thin layer chromatographic analysis of the ethyl acetate soluble fraction indicated five (5) phyto-constituents, their R_f values and the various colours in 10 % H_2SO_4 (Table 1). The results of fractions obtained from the column chromatography (Table 2) showed five fractions labeled PE1 to PE5 at various solvent systems and their corresponding number of spots (Table 2). Preparative thin layer chromatography was used to separate fraction PE2 to isolate the compound. The compound weighed 15 mg with melting point of 217 – 218°C and a retention factor value of (Rf) of 0.52 (Table 3). The short melting point range is indicative of good purity. Pure compounds have sharp melting point.

Table 1: Thin Layer Chromatography of the Ethyl Acetate Soluble Fraction of *P. Erinaceus*

Ethyl acetate fraction	R_f Values	Colour in 10 % H_2SO_4
1	0.57	Purple
2	0.54	Yellow
3	0.64	Brown
4	0.32	Brown
5	0.16	Pink

Key: $R_f \Rightarrow$ Retention factor

Table 2: Fraction from Column Chromatography of Ethyl Soluble Fraction of *P. Erinaceus*

Fractions	Eluting Solvents	Number of Major Spots
PE1	Hexane; Ethyl acetate(100:0)	0
PE2	Hexane; Ethyl acetate(99:1.0)	2
PE3	Hexane; Ethyl acetate(98:2.0)	4
PE4	Hexane; Ethyl acetate(97:3.0)	4
PE5	Hexane; Ethyl acetate(96:4.0)	3

Table 3: TLC Profile of the Isolated Compound PEB2

Compound Code	Solvent System	Observed No of Spot	R_f Value	Melting Point (°C)
PEB2	Hexane ethyl acetate(9:1& 8:2)	1	0.52	216-218

IR spectrum of the isolated compound PEB**2** (Figure 1) showed broad absorption frequency at 3302.4 cm^{-1} which is typical of a hydroxyl group. A fairly intense bands at 2922.2 cm^{-1} and 2851.4 cm^{-1} that can be assigned to an aliphatic C-H (stretch) was observed. The C=C vibration frequency was observed at 1640.0 cm^{-1}. Other peaks present at 1457.4 cm^{-1}, 1379.1 cm^{-1} and 1036.3 cm^{-1} are due to bending vibrations of alkane, alkenes and C-O stretch of an alcohol respectively (Table 5). The IR absorbance values are in concordance with Silverstein *et al*., (2014).

Table 4: Infra-Red Table of the Isolated Compound PEB2

Serial Number	Frequency cm^{-1}	Band	Functional Groups
1	3302.3	O-H$_{\text{Stretch}}$	Alkanols
2	2922.7	C-H$_{\text{stretch}}$	Alkanes
3	2851.4	CH$_{\text{Stretch}}$	Alkanes
4	1640.0	C=C$_{\text{stretch}}$	Alkanes
5	1459.3	CH(Vibration)	Alkanes
6	1377.3	C-H$_{\text{rocking vibration}}$	Alkanes
7	1036.2	C-O$_{\text{stretch}}$	Alkanols

Table 5: Buchard -Liebermann Test for Triterpenoid on the Isolated Compound PEB2

Isolated Compound	Test	Result	Inference
PEBS	Liebermann Buchard's test	+	Triterpenoid

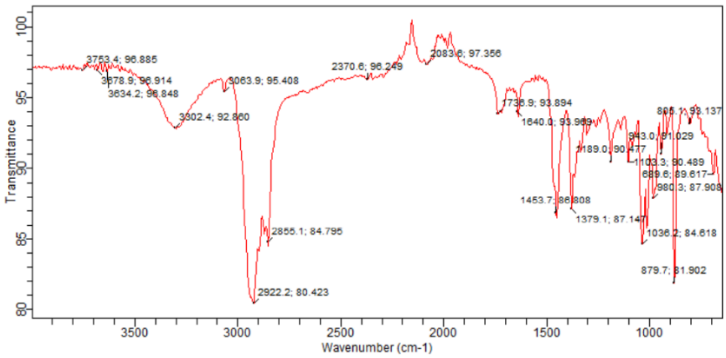

Figure 1: IR Spectrum of Isolated Compound PEB2 showing key Absorption Bands

The mass spectrum was used to analysed and confirm the chemical structure of the compound. The molecular ion (M$^+$) peak of the spectrum was identified at M/Z 426 and it was found to be equal to the calculated molecular weight of the compound lupeol with molecular formula $\mathbf{C_{30}H_{50}O}$. Fragmentation of the molecular ion by removal of a methyl group (– $\mathbf{CH_3}$**)** gave the fragment peak at M/Z 411. This peak is characteristic of a pentacyclic triterpene that has an isopropenyl group (Ahlam *et al.,* 2011; Noor *et al.,* 2019). When the molecular ion M$^+$ losses both water and a methyl group (M$^+$- CH_3-H_2O) it gives the peak at M/Z 393 (Jasiem, 2018). The peak at M/Z 411 fragments by losing ethene ($\mathbf{CH_2{=}CH_2}$) gas to give the fragment at M/Z 383 (Carvalho *et al.,* 2010). Fragment with M/Z 383 further loses $\mathbf{-C_{13}H_{22}}$ and

-H_2O to give fragments with M/Z 207 and M/Z 189 respectively (El Sayed, 2016). These fragments may also arise due to the cleavage between C-8/C-14 and C-12/C-13 bonds (with proton transfer) and is usually a confirmation that such a compound possess a lupane skeleton (Noor *et al.*, 2019). The fragments at M/Z 189 and 218 indicate that compound **PEB2** is a pentacyclic triterpene. Other fragments at M/Z 43, 81, 107, 147, 175, 257 and 315 are also associated with lupeol (Kiria, 2018; Leite *et al.*, 2020). The results of characterization with MS based Library: NIST14.Lib data and comparison with the cited literatures lead to the suggestion of the compound **PEB2** as lupeol. The compound also gave a positive test using Buchard -Liebermann test for triterpenoid (Table 5). The structure of the isolated compound (Lupeol) and it mass spectrum is as shown in figure 2. In this study, the presence of lupeol, a potent bioactive compound in *Pterocarpus erinacius* has been reported for the first time. Lupeol is vastly used as an anti-inflammatory compound. The huge quantity of lupeol makes this plant a future source of lupeol that will help and supports the pharmaceutical industry in drug formulation. Lupeol has been reported to be antiangiogenic, antioxidative and anti-inflammatory in nature (Sudhahar *et al.*, 2008). It inhibits early responses of tumor growth that is induced by benzoyl peroxide (Saleem, 2009). It also plays a very important role in normalizing lipid profile (Sudhahar *et al.*, 2007), wound healing activity (Harish *et al.*, 2008), protective effect in hypercholesterolemia associated with renal damage and suppression of immune factors (Bani *et al.*, 2006).

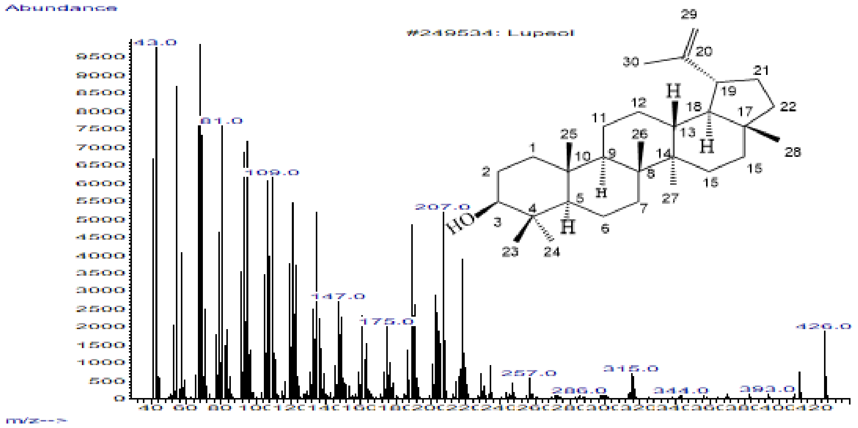

Figure 2: Mass Spectrum of PEB2 and the Proposed Structure: Lupeol

CONCLUSION

From the results obtained, it could be concluded that the root bark of *Pterocarpus erinaceus* contain Lupeol detected by chemical test, Thin Layer chromatography/column chromatography and confirmed by analyses of the data obtained from Gas Chromatography- Mass spectroscopy (GC-MS) and Fourier transformed infrared spectroscopy (FTIR). Lupeol (15 mg) was successfully isolated from the root back extract of *Pterocarpus erinacius* and is reported for the first time. The presence of lupeol in the plant may explain the traditional uses of the root bark of *Pterocarpus erinaceus* to treat cancer, pain, wounds and inflammations. This work present structural elucidation using FTIR and MS data only, work is ongoing to elucidate the structure using ^{1}H and ^{13}C-NMR and the *In-vivo* studies to determine it utilization potential.

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