East African Journal of Health and Science, Volume 8, Issue 1, 2025
Article DOI: https://doi.org/10.37284/eajhs.8.1.2577

East African Journal of Health and Science
eajhs.eanso.org
Volume 8 Issue 1, 2025
Print ISSN: 2707-3912 | Online ISSN: 2707-3920
Title DOI: https://doi.org/10.37284/2707-3920

EANSO
EAST AFRICAN NATURE & SCIENCE ORGANIZATION

Original Article

Extractives from *Vepris glandulosa* Leaf Methanol Extract and Its Antibacterial Activity

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Article DOI: https://doi.org/10.37284/eajhs.8.1.2577

Date Published:
02 January 2025

Keywords:
Vepris glandulosa, Lupeol, Antimicrobial, Crude Extracts Chromatography.

ABSTRACT

The challenges facing currently available antimicrobials such as resistance with no recent significant discoveries are a major health concern in Kenya characterized by prolonged hospitalization, high medical costs, and high annual fatalities. These challenges put great strain on healthcare systems and as such there is emphasis on new drug discoveries to mitigate the predicament. The objective of the study was to determine the antibacterial activity of *Vepris glandulosa* extracts after careful purification, isolation and structural characterization. In this study, crude methanol extracts were obtained using the cold extraction method. Isolation of compounds was accomplished by repeated column and thin-layer chromatography. One compound was obtained which was subjected to both 1D and 2D NMR spectroscopic analysis and the spectroscopic data obtained was used to propose the structure. The compound had its structure fully elucidated and was confirmed by comparing its NMR spectroscopic data with the literature reported to be Lupeol. Lupeol showed antibacterial activity against selected gram-negative and gram-positive bacteria.

APA CITATION
Kirui, D., Kiplimo, J. & Chirchir, D. (2025). Extractives from *Vepris glandulosa* Leaf Methanol Extract and Its Antibacterial Activity. *East African Journal of Health and Science*, *8*(1), 1-9. https://doi.org/10.37284/eajhs.8.1.2577.

CHICAGO CITATION
Kirui, Derrick, Joyce Kiplimo and Denis Chirchir 2025. "Extractives from *Vepris glandulosa* Leaf Methanol Extract and Its Antibacterial Activity". *East African Journal of Health and Science* 8 (1), 1-9. https://doi.org/10.37284/eajhs.8.1.2577

HARVARD CITATION
Kirui, D., Kiplimo, J. & Chirchir, D. (2025). "Extractives from *Vepris glandulosa* Leaf Methanol Extract and Its Antibacterial Activity", *East African Journal of Health and Science*, 8(1), pp. 1-9. doi: 10.37284/eajhs.8.1.2577.

IEEE CITATION
D., Kirui, J., Kiplimo & D., Chirchir "Extractives from *Vepris glandulosa* Leaf Methanol Extract and Its Antibacterial Activity", *EAJHS*, vol. 8, no. 1, pp. 1-9, Jan. 2025.

Article DOI: https://doi.org/10.37284/eajhs.8.1.2577

MLA CITATION
Kirui, Derrick, Joyce Kiplimo & Denis Chirchir "Extractives from *Vepris glandulosa* Leaf Methanol Extract and Its Antibacterial Activity". *East African Journal of Health and Science*, Vol. 8, no. 1, Jan. 2025, pp. 1-9, doi:10.37284/eajhs.8.1.2577.

INTRODUCTION

In Kenya, microbial diseases top the list of causes of death followed by non-communicable diseases according to 2018 research (Ministry of Health: Kenya Cancer Policy, 2019). This is a result of a failure of current antimicrobials in combating the dangers posed by microbial diseases which have developed resistance against them. Projections of impacts of Antimicrobial resistance are 10 million annual human deaths globally by the year 2050 (Ministry of Health: Kenya National Policy on Prevention and Containment of Antimicrobial Resistance, 2017). In recent decades, new drug discovery has been almost non-existent, and synthetic drugs are relatively expensive, most are toxic to the consumer and as a result, there is continued high mortality and morbidity due to failures of existing synthetic antimicrobials (Solowey *et al.*, 2014).

The failures of the available synthetic drugs have created a colossal demand for new drugs during the last decade presenting humanity with the only plausible option of resorting to nature for new drug discovery. Historical records show that plants have been used for their healing potential with great success. This is because natural antimicrobials are readily available, affordable, and non-toxic (Javid *et al.*, 2015).

According to the World Health Organization, it is estimated that 80 % of the earth's population relies on plants as a source of medicine for most of their ailments. The heavy dependence on plants as medicinal sources shows the reliability and effectiveness of plants (Kilonzo & Munisi, 2021). In addition, successful advancements in phytochemistry have renewed interest in herbal medicine (Bishnu *et al*., 2009).

The plant family Rutaceae has been used ethnobotanical for traditional medicine, perfumery, and gastronomy (Tangjitjareonkun & Supabphol, 2014). Rutaceae have a wide range of secondary metabolites which exhibit antibacterial, anti-inflammatory, antifungal, antioxidant, and antiviral, activities (Imbenzi *et al*., 2014). The African continent has roughly 90 species in the *Vepris* genus with notable examples such as *Vepris glandulosa, Vepris nobilis*, *Vepris grandifolia, Vepris hanangensis, Vepris glomerata, Vepris uguenensis* and *Vepris simplicifolia* (Omujal *et al.*, 2020).

The genus Vepris has had great success as an ethno-medicinal source (Imbenzi *et al.*, 2014). The medicinal efficacy of plant materials mostly results from secondary metabolites such as lupeol, flavonoids, phenols, saponins, tannins, and alkaloids (Sivasakthi & Saranraj, 2014). The species in this genus are used in the management of a wide variety of ailments such as lung complications, eye troubles, malaria, coughs and colds, rheumatism, headache, infertility, and as aphrodisiacs (Imbenzi *et al.*, 2014).

Vepris glandulosa is a shrub or tree that grows to 7 m in height. This lower canopy tree is confined to upland dry forests (figure 1). The species grows well in the shade and reproduction is through seeds only. *Vepris glandulosa* is endemic to Kenya and its particular habitat is historically Muguga South Forest Reserve, Ragati, and Limuru which have elevations of over 1550 metres above sea level (Barstow, 2018).

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Figure 1: Picture of the plant *Vepris glandulosa*

As a member of the *Vepris* genus and Rutaceae family, there is a high probability of obtaining secondary metabolites with chemotherapeutic advantages. There have been no earlier phytochemical and biological activity tests done on the leaves of the plant *Vepris glandulosa*.

MATERIALS AND METHODS

Sample collection

The fresh leaves of *V. glandulosa* were collected from Muguga South Forest Reserve and transported to the University of Kabianga Chemistry Laboratory. Identification of the collected sample was done by a Botanist at the Department of Biological Sciences and a voucher specimen was deposited in the Herbarium Voucher No 1530. It was cleaned in running tap water, chopped into smaller pieces then air-dried at room temperature for a period of two weeks. The sample was ground to powder using an electric grinder and stored for further use.

Cold extraction

The maceration technique was used to obtain the crude extracts. About 1.4 Kg of the plant sample was weighed and then soaked with 1.5 L of methanol for a period of exactly 72 hours at standard temperature and pressure with gyration every 12 hours. Crude extract was obtained after decantation then it was concentrated under reduced pressure using a rotary evaporator and left to dry to a constant weight at room temperature.

Isolation of compounds and NMR spectroscopic analysis

The crude extracts were purified using repeated column and thin-layer chromatography. Column Chromatography was carried out by using silica gel 60 mesh size 70-230 (Merck, 0.063-0.200 mm) as a stationary phase. This was done by use of gradient elution using ethyl acetate and *n*-hexane solvent systems. The 1D NMR (^{1}H and ^{13}C NMR), DEPT, and 2D experiments (HSQC, HMBC, COSY, NOESY) of the isolated compounds were analyzed using Nuclear Magnetic Resonance (NMR) spectroscopic method on a Bruker Avance^{III} 400 MHz spectrophotometer at room temperature, using deuterated chloroform ($CDCl_3$). The NMR spectra were obtained and recorded at high resolution and developed using tetramethylsilane (TMS) as an internal reference.

Zone of inhibition test

The bacteria chosen for the assay based on availability were American Type Culture Collection (ATCC 29213) *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The Agar well diffusion method was used. Preparation of the Mueller Hinton agar was done following the manufacturer's

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instructions on the label of the bottle. The standard antibiotic used as the control was Amoxicillin.

The 8 Petri dishes were each streaked with bacteria with 4 dishes for each of the 2 bacterial colonies. Compound 1 was prepared for impregnation by mixing with a small amount of methanol and then shaken vigorously to form a solution. The 6mm disks were then soaked in the solution and then placed onto the prepared petri dish with the Mueller Hinton agar. The disks with compound 1 were placed on 6 of the petri dishes meaning 3 out of 4 petri dishes for each bacteria had compound 1. The remaining 2 disks were for the Amoxillin that was placed one for each bacterial colony.

The 8 petri dishes were put inside an incubation chamber set at 37^{o}C and measurements were recorded after 48 hours.

Minimum Inhibitory Concentration test

The procedure began with the assembly of 10 clean sterile test tubes which were placed on 2 test tube racks with five test tubes for each rack. Five test tubes were used for *Pseudomonas aeruginosa* and the remaining five for *Staphylococcus aureus.* All ten test tubes were put in 10ml of distilled water but then the addition of a solution of compound 1 with varying volumes was done on 8 out of the 10 test tubes. Of the 8 test tubes, two test tubes were put in 2ml, another two 4ml, another two 6ml, and the last two 8ml.

The ten test tubes were each inoculated with bacteria, five for *Pseudomonas aeruginosa* and five for *Staphylococcus aureus* then allowed time to grow. Observations were made after 24 hours. One out of each 5 pairs of test tubes was not added compound 1 so as to act as the positive controls.

Minimum Bactericidal Concentration

Results from the minimum inhibitory concentration test were to used determine the parameters for the minimum bacterial concentration test. The concentration preceding the minimum inhibitory concentration was chosen as the lowest concentration for the assay for the two bacteria types of *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Eight sterile petri dishes with Mueller Hinton agar were used for the assay and four petri dishes were streaked with *Staphylococcus aureus* and another four with *Pseudomonas aeruginosa*. The streaked petri dishes were impregnated with varying concentrations of compound 1 and then put in the incubator for 24 hrs to allow bacterial growth. The positive control was the concentration of the preceding minimum inhibitory concentration.

RESULTS AND DISCUSSIONS

Compound 1 was isolated as a crystalline white solid with a threaded crystal-like appearance. The ^{1}H NMR spectrum of compound 1 showed seven methyl singlets at δ_H 0.70, 0.76, 0.82, 0.96, 0.98, 1.02, 1.68, and one secondary hydroxyl group as a doublet of doublets at δ_H 3.20. It also showed two olefinic protons at δ_H 4.57 and 4.68 representing the exocyclic double bond. ^{1}H NMR ($CDCl_3$, 400 MHz) δ:4.57 (s, H-29a), 4.68 (s, H-29b), 3.20 (1H, dd, J = 5.12; 11.27 Hz, H-3),1.68, 1.02, 0.98, 0.96, 0.82, 0.76, 0.70 (21 H, 7 s, 7 CH_3).

The ^{13}C - NMR spectrum of the compound indicated 30 carbon signals (seven methyl, eleven methylene, six methine and six quaternary carbons) for the terpenoid of the lupane skeleton which includes a carbon bonded to the OH group at the C-3 position that appeared at δ_C 79.01 ppm. The olefinic carbons of the exocyclic double bond appeared at δ_C 150.95 ppm (quaternary C) and δ_C 109.33 ppm (methylene C) which are assigned as C-20 and C-29 double bond of the lupane type triterpenoid compound. Complete assignment of all protons and carbons was confirmed by ^{1}H-^{1}H COSY and long-range signals in HMBC spectra. Therefore, the structure was assigned the name Lupeol (3-lup-20(29)-en-3-ol). Table 1, an assessment of similarities between the compound and that reported by Shwe *et al*. (2019) showed the chemical shift at C-3 is at 79.01 while that of Shwe *et al*. (2019) the C-3 is at 81.2 which is very close

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to each other. They both have an OH at the C-3 thus also heavily implying that the unknown compound is also a Lupeol.

Table 1 .^{13} CNMR spectroscopic results for the pure isolated compound

Number	The chemical shift of compound 1 $^{\delta}$C	Chemical shift of Lupeol (Shwe *et al.*, 2019)
C-1	37.16	38.85
C-2	27.40	27.40
C-3	79.01	79.01
C-4	38.71	38.69
C-5	55.29	55.28
C-6	18.00	17.99
C-7	34.28	34.26
C-8	42.82	40.82
C-9	50.43	50.42
C-10	35.58	37.16
C-11	20.93	20.92
C-12	25.13	25.12
C-13	38.05	38.04
C-14	43.00	42.82
C-15	27.44	27.40
C-16	40.00	35.57
C-17	40.83	47.98
C-18	47.98	48.29
C-19	48.30	47.98
C-20	150.95	150.99
C-21	20.84	29.83
C-22	38.85	39.99
C-23	27.99	27.98
C-24	15.37	15.36
C-25	16.12	16.11
C-26	15.97	15.96
C-27	14.55	14.54
C-28	18.32	18.31
C-29	109.33	109.31
C-30	19.31	19.29

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Figure 2: CNMR spectroscopy results for compound 1

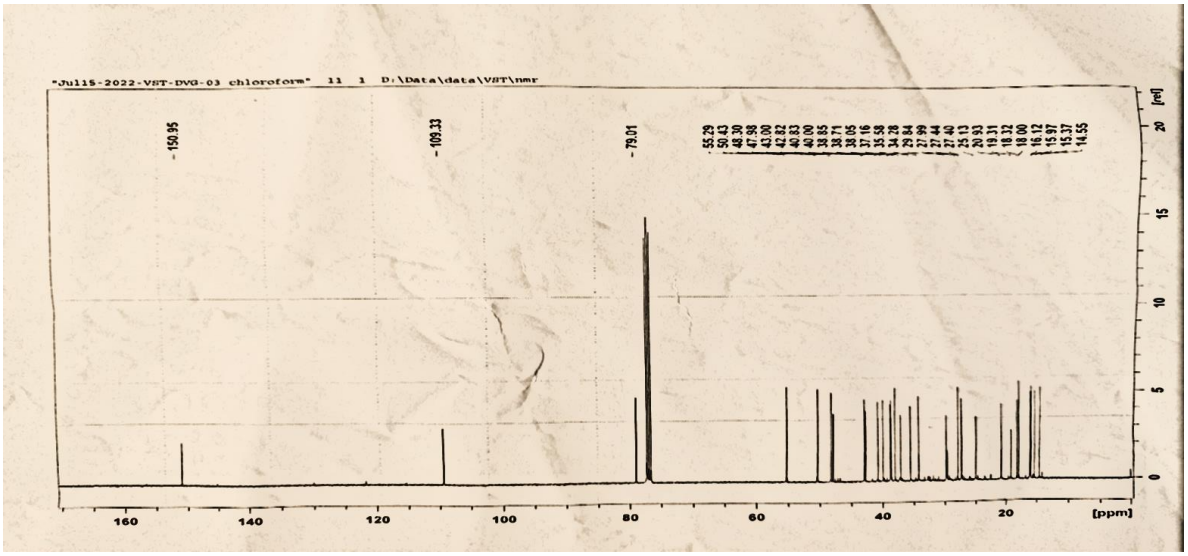


Figure 3: HNMR results for compound 1

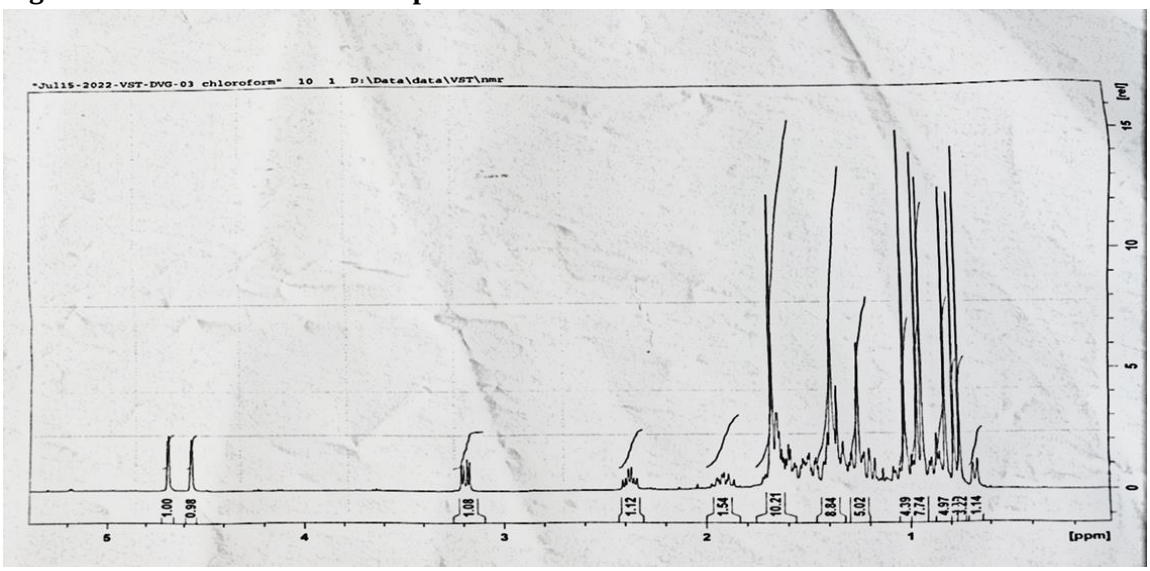
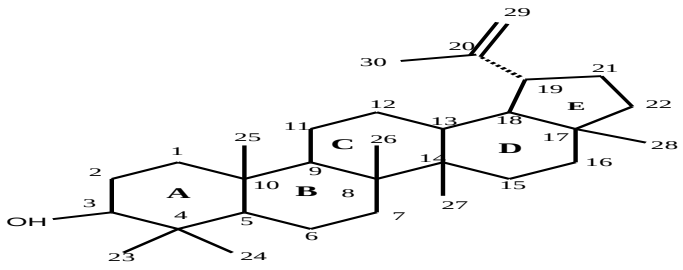


Figure 4: Proposed structure of compound 1 identified as Lupeol



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Antibacterial activity assays for compound 1

Zone of Inhibition

The commercial amoxicillin showed sensitivity against *S. aureus* and *P. aeruginosa* in ascending order of 20.00 mm and 40.00 mm respectively. For compound 1, resistance was shown by *Pseudomonas aeruginosa* at an 11.00 mm zone of inhibition but fortunately, it showed moderate sensitivity against *Staphylococcus aureus* with a zone of inhibition of 13.66 mm.

Table 2. Zone of inhibition table of results, comparison and classification.

Antimicrobial	Zone of inhibition in millimetres (mm)		
	Resistant	Moderately sensitive	sensitive
Amoxicillin zone of inhibition ranges (Sarker *et al.*, 2014)	≤ 13	14-17	≥ 18
Amoxicillin against *S. aureus*			20.00
Crude extract against *S. aureus*		13.66	
Amoxicillin against *P. aeruginosa*			40.00
Crude extract against *P. aeruginosa*	11.00		

Minimum Inhibitory Concentration test

Results of the Minimum Inhibitory Concentration assay done on *Pseudomonas aeruginosa* and *Staphylococcus aureus* are shown in Table 3. At 2 µg/ml and 4 µg/ml concentrations, compound 1 was unable to inhibit the growth of the *Pseudomonas aeruginosa* bacterial colonies, but the 6 µg/ml and 8 µg/ml had no visible signs of bacterial growth thus those concentrations were sufficient to stop the growth of *Pseudomonas aeruginosa* colonies. The 6 µg/ml concentration is the minimum inhibitory concentration of the crude extract against *Pseudomonas aeruginosa.*

At 2 µg/ml concentration, the compound was unable to inhibit the growth of the *Staphylococcus aureus* bacterial colonies, but the 4 µg/ml, 6 µg/ml and 8 µg/ml had no visible signs of bacterial growth thus those concentrations were sufficient to stop the growth of *Staphylococcus aureus* colonies. The 4 µg/ml concentration is the minimum inhibitory concentration of the crude extract of *Vepris glandulosa* leaves against *Staphylococcus aureus.*

Table 3: Minimum inhibitory concentration results

Bacteria	Concentration of crude extract µg/ml	Observation	Conclusion
Pseudomonas aeruginosa	2	Growth	Insufficient concentration as the bacterial growth inhibitor
	4	Growth	Insufficient concentration as the bacterial growth inhibitor
	6	No growth	Minimum inhibitory concentration
	8	No growth	Sufficient concentration as the bacterial inhibitor
	0 (positive control)	Growth	Complete absence of crude extract
Staphylococcus aureus	2	Growth	Insufficient concentration as the bacterial growth inhibitor
	4	No growth	Minimum inhibitory concentration
	6	No growth	Sufficient concentration as the bacterial growth inhibitor
	8	No growth	Sufficient concentration as the bacterial growth inhibitor
	0 (positive control)	Growth	Complete absence of crude sample extract

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Minimum Bactericidal Concentration test

At 2 µg/ml 4 µg/ml and 6 µg/ml concentrations, the compound was unable to kill the *Staphylococcus aureus* bacterial colonies, but the 8 ml had no visible signs of bacterial growth thus it was sufficient as a bactericide for *Staphylococcus aureus* colonies. The 8 µg/ml concentration is the minimum bactericidal concentration of the crude extract of *Vepris glandulosa* leaves against *Staphylococcus aureus.* At 4 µg/ml and 6 µg/ml concentrations, the crude extract was unable to kill the *Pseudomonas aeruginosa* bacterial colonies, but the 8µg/ml had no visible signs of bacterial growth thus it was sufficient as a bactericide for *Pseudomonas aeruginosa* colonies. The 8 µg/ml concentration is the minimum bactericidal concentration of the crude extract against *Pseudomonas aeruginosa*.

Table 4. Minimum Bactericidal Concentration experimental results

Bacteria	Concentration of crude extract µg/ml	observation	conclusion
Staphylococcus aureus	2 (positive control)	Bacterial growth	Insufficient concentration as the bactericide
	4	Bacterial growth	Insufficient concentration as the bactericide
	6	Bacterial growth	Insufficient concentration as the bactericide
	8	No bacterial growth	Minimum bactericidal concentration
Pseudomonas aeruginosa	4 (positive control)	Bacterial growth	Insufficient concentration as the bactericide
	6	Bacterial growth	Insufficient concentration as a bactericide
	8	No growth	Minimum bactericidal concentration

CONCLUSION

The isolated purified compound **1** was successfully identified as Lupeol based on the NMR spectroscopic data. The antimicrobial activity of the crude extract from the leaves of *Vepris glandulosa* was evident and may not be strongly recommended for clinical trials based on the zone of inhibition test in comparison with commercial Amoxicillin. On the other hand, the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration test results suggested that *Vepris glandulosa* may be considered as a potential option as an antimicrobial drug because of having bacteriostatic and bactericidal activity.

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