Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article
Volume 13, Issue 4, 2024, 185
https://doi.org/10.33263/LIANBS134.185

Hardwickiic acid and Lupeol from Leaves of *Croton macrostachyus* and their Antibacterial Activity

Sisay Awoke [1], Liliy Kumsa [1]

[1] Department of Chemistry, College of Natural Sciences, Wollo University, Dessie, Ethiopia; P.O. Box 1145, sisayawoke2013@gmail.com (S.A.); liliykumsa2015@gmail.com (L.K.);
* Correspondence: sisayawoke2013@gmail.com;
Scopus Author ID 58174900300
Received: 25.07.2023; Accepted: 8.07.2024; Published: 28.08.2024

Abstract: Croton macrostachyus, one of the Croton species found in Ethiopia, is used traditionally for the treatment of various health ailments, including diabetes, malaria, stomachache, ascariasis, abdominal pain, gonorrhea, wounds, ringworm infestation, and hemorrhoids. The main objective of this study was to isolate and characterize compounds from the *C. macrostachyus* leaves and determine their antibacterial activities. Maceration was used to extract the plant material for isolating compounds, and the column chromatography technique was applied. The antibacterial activities of the crude leaf extracts, obtained using petroleum ether, chloroform, and methanol, were determined by measuring the diameters of the zone of inhibition. Phytochemical screening of methanol, chloroform, and petroleum ether extract from leaves revealed the presence of terpenes, flavonoids, alkaloids, and saponins. Furthermore, the in vitro antibacterial activity of leaf extracts against Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, and Listeria monocytogenes will be investigated using the agar plate disc diffusion technique. The maximum inhibition zone (15.83 ± 0.22 mm) was recorded in methanol extracts against *E. coli* at a 150 mg/mL concentration. All concentrations of the crude extract, except for petroleum ether extract at a concentration of 50 mg/mL against *S. aureus* (8.69 ± 0.25 mm), showed higher inhibition than positive control against tested pathogens. Antibacterial-guided fractionation of the methanol extract of the leaves was subjected to column chromatography on silica gel, and preparative TLC subsequently isolated selected fractions to give the two compounds: lupeol and hardwickiic acid. Their structures were elucidated based on spectral studies and compared with published data. Lupeol and Hardwickiic acid were evaluated for their antibacterial activities. The isolated compounds displayed significant antibacterial activity but less than crude extracts.

Keywords: croton; macrostachyus; antimicrobial; hardwickiic; lupeol.

© 2024 by the authors. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Natural product chemistry is the chemistry of naturally occurring organic compounds: their biosynthesis, environmental function, and metabolism. It is a part of organic chemistry that covers more conventional branches of chemistry, such as structural elucidation and synthesis [1]. In plants, there are compounds that serve important biological and ecological roles fundamentally as chemical messengers and for defense purposes known as secondary metabolites. They are of limited occurrence and no apparent utility, whereas primary metabolites are essentially ubiquitous and certainly essential for life [1].

In ancient times, natural products were used to cure human diseases and were as universal as medicine. The currently available drugs for treating different human and animal diseases come from thousands of medicinal plants [2]. Such medicines have been discovered after herbalists observed the medicinal use of particular plants and the subsequent isolation of bioactive compounds from the plant or part traditionally used for treating different human illnesses. Moreover, some compounds have also been used as precursors that can be modified synthetically to improve their therapeutic activities [3].

To screen, isolate, and identify potential drug compounds quickly and precisely from natural sources to alleviate human illnesses, several new and highly specific in vitro bioassay techniques, chromatographic methods, spectroscopic techniques, and other standardized pharmacological methods have also been introduced and developed [1]. There are several reasons that necessitate the isolation and characterization of bioactive compounds from natural sources [4]. Since the number of bioactive compounds gained from medicinal plants is very small, they can be prepared synthetically without relying on plants once such compounds are known and their properties and structures are determined. Otherwise, many plants should be destroyed to harvest significant quantities of such compounds for disease treatment [5].

The increasing resistance of bacteria became apparent following the development of new drugs to delay, prevent, or treat infectious diseases [6]. Plants are the best candidates for antimicrobial agents to combat infectious diseases since they have been used for centuries without significant resistance development [7]. The primary extraction methods are very variable, but the idea is to investigate activities cited in popular use and to choose the same extraction method. Quite a number of studies have been conducted to confirm the screening of antimicrobial compounds from higher plants [8-10]

C. macrostachyus is one of the plant species that is known for its medicinal use. It is native to some eastern African countries, such as Eritrea, Ethiopia, Kenya, Tanzania, Uganda, and Nigeria. It is one of the eight Croton species found in Ethiopia [11,12]. The plant is used for the treatment of several human health problems in areas where it is native, which include the symptoms of diabetes [13,14], malaria [15,16], dysentery and stomachache, ascariasis and taeniasis [17], abdominal pain [18], gonorrhea, wounds [19], ringworm, infestation, hemorrhoids [20], venereal diseases and cough [21]. In Ethiopia, the plant also has folk medicinal uses as a purgative for various skin diseases, the management of helminths and venereal diseases, and to induce abortion [22]. This wide range of medicinal uses of *C. macrostachyus* led scientists to isolate compounds from its different parts. Some compounds isolated from different parts of the plant have been tested for their biological activities [23,24]. Different research teams are still carrying out scientific investigations to examine the biological activities of crude extracts or pure compounds isolated from C. macrostachyus. Most of them showed promising activities [25,26].

In Ethiopia, a few studies have been conducted, mainly emphasizing the antibacterial activities extracted from *C. macrostachyus* leaves. However, papers on the antibacterial activity of isolated compounds from the leaf extract are rare. Therefore, this study aimed to isolate compounds and determine the antibacterial activities of extracts and pure isolated compounds from C. macrostachyus leaf extracts.

2. Materials and Methods

2.1. Chemicals and apparatus.

Solvents, such as ethanol, methanol, ethyl acetate, chloroform, hexane, and petroleum ether, were used for extraction. Chemicals and reagents used for phytochemical investigation were distilled water, burette reagent, sodium hydroxide, ferric chloride, concentrated sulfuric acid, acetic acid, diethyl ether hydrochloric acid, amyl alcohol, and DMSO. All chemicals and reagents used in this study were imported from CDH P. Ltd., New Delhi, India (ISO 9001). Chloramphenicol was used as a standard positive control. The following materials and instruments were used during the experiment: thin-layer chromatography made from pre-coated silica gel on alumina, test tubes, funnels, beakers, filter paper, hot plates, oven, rotary evaporator attached with a vacuum (model: YC7124, Beijing, China), electronic balance (1810-BA Model, Beijing, China), grinder, water bath (Re201BL, Model Indian), petri dish, micropipettes, aluminum foil, measuring cylinder, refrigerator (Samsung model RT34SUMG, Thailand), incubator (DHP-9052B Model), separatory funnel, swab, McFarland densitometer (Den-18 MC model), water distiller, sterilizer. NMR-spectra measurements were performed with the Bruker ACQ 400 AVANCE spectrometer operating at 400 MHz in $CDCl_3$. The infrared spectra were recorded using a Perkin-Elmer BX Spectrometer (400–4000 cm^{-1}) in KBr. UV data were recorded using a T60 UV-VS spectrophotometer in MeOH. The molecular weight of isolated compounds was confirmed using a mass spectrometer. Optical rotations were measured on a Rudolph Autopol VI Automatic polarimeter (Hackettstown, NJ, USA).

2.2. Collection of plant material and extraction.

Plant material: The wild leaves of *C. macrostachyus* were collected in September 2020 from the Amhara region of the North Shewa zone, Ataye town, located at a distance of 270 km from the capital city of Addis Ababa, Ethiopia, following the guidelines proposed by Wondafrash [27]. The plant was collected after getting written consent from the local authority and a special letter from the Wollo University Postgraduate Office. The specimen was submitted to Addis Ababa University (AAU), Addis Ababa, Ethiopia, and the National Herbarium of Ethiopia for identification and voucher numbers. The sample was authenticated by Prof. Sebsebe Demissew and Ato Melaku Wondafrash, and voucher number AAU-Herbarium-272813 for *C. macrostachyus* was deposited at the National Herbarium of Addis Ababa University, Ethiopia.

The freshly collected leaves samples were washed with water to eliminate dust and other debris. They were then spread out to dry at room temperature and subsequently ground into a fine powder using a mortar. The obtained powder was stored in an airtight container and kept refrigerated at 4°C until it was ready for analysis. For the extraction process, 100 g of the ground leaves were soaked separately in 300 mL of different solvents (petroleum ether, chloroform, and methanol) and left on a platform shaker for approximately 72 hours. The mixture was then filtered using Whatman No.1 filter paper. The petroleum ether, chloroform, and methanol extracts were dried using a rotary evaporator to obtain the crude extract. The dried extracts were stored in labeled sterile screw-capped bottles at 5°C in airtight vials until they were ready for use [28].

2.3. Phytochemical Screening.

Common secondary metabolites were detected for methanol, chloroform, and petroleum ether leaf extract of *C. macrostachyus* using the preceding analytical procedures.

2.3.1. Terpenes test.

250 mg of ethanol extract was mixed with 2 mL of $CHCl_3$, and 30 mL of concentrated H_2SO_4 was added carefully to form a layer. The reddish-brown coloration of the interface was inspected [29,30].

2.3.2. Flavonoids test.

250 mg of ethanol extract was dissolved in a small amount of dilute NaOH, and concentrated HCl (3 mL) was added. A yellow solution that turns colorless was inspected [30,31].

2.3.3. Tannins test.

A small quantity of the ethanol extract was mixed with water and heated in a water bath. The mixture was filtered, and a small amount of solid $FeCl_3$ was added to the filtrate. Dark-green solution was inspected [32].

2.3.4. Alkaloids test.

250 mg of the crude extract was mixed with 2 mL of concentrated hydrochloric acid. The mixture was then filtered and mixed with a small amount of amyl alcohol at room temperature. The mixture was kept to observe the color of the alcoholic layer [33].

2.3.5. Saponins test.

250 mg of the ethanol extract was shaken with 5 mL of distilled water for 30 min and then heated to a boil. The appearance of a creaming mix of small bubbles (frothing) was inspected [34].

2.3.6. Anthraquinones test.

500 mg of the ethanol extract was boiled with concentrated hydrochloric acid for a few minutes in a water bath and filtered. The filtrate was allowed to cool, and an equal volume of $CHCl_3$ was added to it. A few drops of ammonia were added to the mixture and heated in a water bath. The formation of rose-pink color was inspected [35].

2.3.7. Phenol test.

The extract (500 mg) was dissolved in 5 mL of distilled water. A few drops of neutral 5% ferric chloride solution were added to this. A dark green color indicates the presence of phenolic compounds [36].

2.3.8. Glycosides test.

A small amount of the methanol extract of *A. integrifolia* was added to 1 mL of water. A few drops of sodium hydroxide (NaOH) solution were added [37].

2.4. Isolation of compounds from methanol extract.

From the methanol extract, 10 g was mixed with silica gel by methanol to adsorb the crude extract for column chromatography [38]. After drying using a rotary evaporator, black solid extract was obtained, and then the column was packed with silica gel and hexane [39]. After one hour, the adsorbed sample was applied to the top of the packed column, and then the column was eluted using different solvent systems with increasing gradients [40,41]. By this similar step, many fractions were collected, and the TLC of each fraction was checked using a different ratio, n-hexane: ethyl acetate. Fractions that have similar spots (similar *Rf*) were then mixed and concentrated by a rotary evaporator to obtain 22 fractions. Fractions 16 and 19 were observed as promising fractions and then applied on preparative TLC for further purification to obtain 910 mg and 760 mg, respectively. Finally, these fractions showed characteristic single-colored spots on TLC upon using a UV lamp at wavelengths of 254 nm and 366 nm after spraying 1% vanillin sulfuric acid and heating for a few minutes. The resulting purified samples were submitted to spectroscopic analysis and fraction 16 and 19 were characterized as hardwickiic acid and lupeol [42].

2.5. Antibacterial activity.

2.5.1. Preparation of stock solution of the extracts.

The stock solutions (petroleum ether, chloroform, and methanol extracts of *C. macrostachyus* and two isolated compounds*)* were prepared by dissolving 0.5 g of each of the extracts with dimethyl sulfoxide (DMSO) until the volume of the solution was 1 mL. Different concentrations (50, 100, and 150 mg/mL) of each of the extracts were then prepared from their respective stocks upon dilution, using DMSO as diluents [43]. The positive control chloramphenicol was prepared by dissolving 45 mg in 3 mL of DMSO. The negative control was 5% DMSO (*v*/*v*).

2.5.2. Test organisms.

The selected human pathogenic bacteria, *S. aureus*, *K. pneumonia*, *L. monocytogenes,* and *E. coli*, were obtained from the Department of Biology, Wollo University, Dessie, Ethiopia. The bacteria were maintained on nutrient agar and stored in the refrigerator at 4°C. All the selected bacterial strains were cultured on nutrient agar plates and then incubated for 24 h at 37°C [44]. Some of the colonies from these cultures were inoculated into Mueller–Hinton broth and incubated at 37°C for 24 h before use [45].

2.5.3. Standardization of inoculums.

The colonies formed were picked up with a sterile inoculating loop, transferred into a test tube containing 4 mL of sterile normal saline, and vortexed thoroughly. This was repeated until the turbidity of each bacterial suspension matched the turbidity of the 0.5 McFarland

standards, as the resulting suspension was used as inoculums for the test pathogen to be used in the antibacterial susceptibility test [46,47].

2.5.4. In vitro antibacterial assay.

The antibacterial activities of the crude extracts leaves of petroleum ether, chloroform, and methanol extracts of *C. macrostachyus* were determined by measuring the diameters of the zone of inhibition. Each dried plant extract was dissolved and used to impregnate the paper discs, as described by [48]. The suspensions were uniformly distributed on the MHA surface by sterile cotton swabs. The plant extract-impregnated discs (50, 100, and 150 mg/mL) were then applied on the inoculated MHA plates using sterile forceps at approximately equidistant positions to each other and allowed to stand for 30 min at room temperature to permit proper diffusion of the extract. Finally, the plates were incubated for 24 h at 37°C to ensure robust cell growth. After incubation, the diameter of the zone of inhibition was measured in millimeters using a ruler for each extract against each pathogen. The experiments were done in triplicates, and all antibacterial activities were expressed as mean diameters (mm) of inhibition zones produced by the plant extract [49].

3. Results and Discussion

3.1. Percentage yields and phytochemical screening tests of extracts.

The yield of the crude methanol, chloroform, and petroleum ether of leaves extract of *C. macrostachyus* is presented in Table 1. The methanol crude extract had the highest percentage yields compared to chloroform and petroleum ether crude extract, but petroleum ether had the lowest percentage yields, as shown in Table 1. The result of the percent yield shows that the test plant contains more polar constituents than nonpolar ones.

Table 1. The percentage yield of the crude extracts.

Types of Extracts	Percent Yield
Petroleum ether	5.32
Chloroform	6.10
Methanol	12.50

The phytochemical screening tests were tabulated, as shown in Table 2. Alkaloids, terpenoids, flavonoids, and saponins were found in petroleum ether, but tannins, phenols, glycosides, and anthraquinones were not noticed. Alkaloids, terpenoids, flavonoids, and saponins were found in all the extracts, but anthraquinones were absent. In agreement with this, it was reported that the methanolic leaf extract of *C. macrostachyus* contained secondary metabolites such as tannins, alkaloids, terpenoids, saponins, and flavonoids [10]. They also reported that the ethanolic *C. macrostachyus* leave extracts contained alkaloids, terpenoids, tannins, and flavonoids.

Table 2. Phytochemical screening tests of methanol, chloroform, and petroleum ether extracts.

Secondary metabolites	Petroleum ether	Chloroform	Methanol
Alkaloids	+	+	+
Terpenoids	+	+	+
Saponins	+	+	+
Flavonoids	+	+	+
Glycosides	−	+	+

Secondary metabolites	Petroleum ether	Chloroform	Methanol
Phenols	−	+	+
Tannins	−	−	+
Anthraquinones	−	−	−

(−) not detected; (+) detected

3.2. Characterization of lupeol.

Lupeol, $C_{30}H_{50}O$, was obtained as a white crystal with a melting point range of 214–217°C. A TLC chromatogram results in a violet spot at *Rf* = 0.43 when developed with hexane: ethyl acetate (4:1) solvent system. An absorption maximum at 272 and 320 nm appeared in the UV-Vis spectrum. A molecular ion peak at *m/z* 426 and relevant fragment ions at *m/z* 189 and 207 were observed at the EIMS, which are considered to be characteristic of the fragmentation of triterpenes with a lupane skeleton bearing a hydroxyl group at C-3 [50]. In the IR spectrum, bands at 3440 cm^{-1} and 1638 cm^{-1} were very intense absorption characteristic signals exhibited by lupeol due to a hydroxyl group corresponding to C=C bonds. The ^{1}NMR data of lupeol designated the oxymethine proton signal appeared as a doublet of doublets at δ 3.20 ppm (1H, *dd*, *J* = 11.2, 5.0 Hz, H-3), and this splitting pattern has been used to differentiate between lupeol and epilupeol (δ 3.40, *t*, H-3) [50].

The oxygenated carbon signal in the ^{13}C NMR spectrum of lupeol appeared at δ 79.0, which is more deshielded than the C-3 signal in epilupeol (δ 76.0 ppm). The other important signal that supports this fact is the chemical shift of C-5. The C-5 resonance appeared at δ 55.3 ppm in lupeol, which is more deshielded than the corresponding signal in epilupeol (δ 49.0 ppm). The orientation of a typical oxymethine proton was judged on the basis of facts in the literature. The proton at C-3 (H-3) resonances appeared at 3.20 ppm in lupeol, which indicated that the hydroxyl groups at C-3 should have β-orientation, which differentiates these triterpenes from their corresponding *epimer*, epilup ol. Thus, the OH group at C-3 in lupeol must be β-oriented (Figure 1) [51]. The spectroscopic data were in excellent agreement when compared with the literature data, as shown in Table 3 [51].

3.3. Characterization of hardwickiic acid.

Hardwickiic acid, $C_{20}H_{28}O_{3}$, was obtained from white powder. The optical rotation of the isolated compound was [D] 25 −35°. A light blue spot was observed on TLC at *Rf* = 0.38 with the solvent system of hexane: ethyl acetate (4:1). The UV-Vis spectrum showed absorption maximum at λmax 210 nm. The EIMS showed a molecular ion peak at *m/z* 317.2. The IR spectrum revealed that the medium abortion peak at 3397 cm^{-1} indicated the presence of acidic O-H stretching, and the other medium signal at 1684 and 1640 cm^{-1} showed the C=C bond stretching. Moreover, the more intense signals that appeared at 1257 and 1078 cm^{-1} were indicative of C-O stretching; also, the more intense peak between 2958–2864 was due to C-H stretching.

In the ^{1}H NMR data, four olefinic protons were detected at a chemical shift of δ 7.18, 7.32, 6.23, and 6.82 ppm. The most deshielded triplet signal appeared at δ 7.32 ppm (1H, *t*, *J* = 2.0 Hz, H-15) due to oxygenated carbon at C-15 and, similarly, a proton attached at C-16 appeared at δ 7.18 ppm (1H, *b*s, H-16) as a broad singlet signal. Two doublets of doublets signals appeared at δ 6.23 ppm (1H, *dd*, *J* = 2.0, 1.0 Hz, H-14) and 6.82 (1H, *dd*, *J* = 5.0, 3.0 Hz, H-3) were assigned for C-14 and C-3, respectively. The ^{13}C NMR spectra revealed five quaternary carbons, of which two were olefinic, two aliphatic, and one carbonyl carbon. The

most deshielded carbon appeared at 172.2 ppm due to the carboxylic acid functional group. Six olefinic carbons were recorded at a chemical shift of δ 110.9, 125.4, 138.2, 140.1, 141.3, and 142.6 ppm, which were assigned for C-14, C-13, C-16, C-3, C-4 and C-15, respectively.

The ^{13}C-NMR spectra indicated the presence of twenty signals with similar characteristics to those of the clerodane diterpene skeleton [52,53] and displayed the presence of three methyl groups at δ 15.9 ppm (C-17), δ 20.4 (C-19) and δ 18.1 (C-20). IR spectra displayed at 3397 cm^{-1} and 1684 cm^{-1}, UV absorption at λmax 210 nm, and the signals observed in the ^{13}C-NMR spectra at δ 172.4 ppm (C-18), 140.1 ppm (C-3) and 141.3 ppm (C-4) were indicative of an α, β-unsaturated carboxylic group. Moreover, the ^{1}H and ^{13}C NMR spectra showed signals that could be attributed to a β-mono-substituted furan ring in the lateral chain of the diterpene. The complete analysis of the NMR signals (Table 3) was in agreement with the NMR data from the literature for hardwickiic acid [54,55]. The negative value of [α] D25 confirmed the absolute configuration as (−)-hardwickiic acid (Figure 1). The compound was previously isolated from this species.

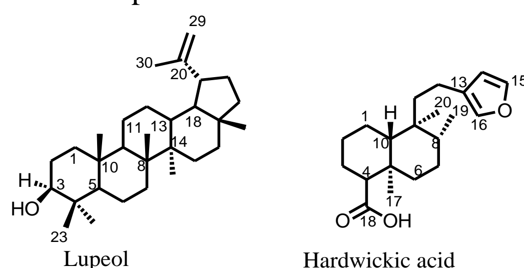

Figure 1. Structures of Lupeol and hardwickiic acid.

Table 3. ^{13}C NMR data for lupeol and hardwickiic acid and their literature (400 MHz, $CDCl_3$).

CN	Lupeol		Hardwickiic acid		CN	Lupeol		Hardwickiic acid	
	Expt.	*Lit. [56]*	*Expt.*	*Lit. [57]*		*Expt.*	*Lit.*	*Expt.*	*Lit.*
1	38.7	38.7	17.3	17.5	16	35.5	35.5	138.2	138.4
2	27.4	27.4	27.1	27.3	17	43.0	43.0	15.9	16.0
3	79.0	79.0	140.1	140.2	18	48.3	48.3	172.4	172.5
4	38.8	38.9	141.3	141.5	19	47.9	48.0	20.4	20.6
5	55.3	55.5	37.4	37.6	20	150.9	151.0	18.1	18.3
6	18.3	18.5	35.7	35.9	21	29.8	29.9	-	-
7	34.2	34.2	27.4	27.5	22	40.0	40.0	-	-
8	40.8	40.9	36.2	36.3	23	28.0	28.0	-	-
9	50.4	50.5	38.8	38.9	24	15.3	15.5	-	-
10	37.1	37.2	46.5	46.7	25	16.1	16.1	-	-
11	20.9	21.0	38.4	38.7	26	15.9	16.0	-	-
12	25.1	25.2	18.1	18.2	27	14.5	14.8	-	-
13	38.0	38.1	125.4	125.6	28	18.0	18.0	-	-
14	42.8	42.9	110.9	111.0	29	109.3	109.0	-	-
15	27.4	27.1	142.6	142.7	30	19.3	19.5	-	-

3.4. Antibacterial activity.

The values in Table 4 were the mean of triplicate values in the form of the mean plus the standard error of the mean. As shown in the table, at a concentration of 50 mg/mL, the zone of inhibitions of the petroleum ether extracts of *C. macrostachyus* leaf against *S. aureus*, *L. monocytogenes*, *K. pneumoniae,* and *E. coli* was 9.09 ± 0.74, 8.26 ± 0.06, 9.08 ± 0.13, and 9.59 ± 0.12 mm, respectively. Similarly, at a concentration of 50 mg/mL, the zone of inhibitions of the chloroform extracts of *C. macrostachyus* leaf against *S. aureus*, *L. monocytogenes*, *K. pneumoniae* and *E. coli* was 8.69 ± 0.25, 9.18 ± 0.30, 9.35 ± 0.28, and 11.05 ± 0.42 mm,

respectively. At the same concentration, the zone inhibition of methanol extract of this plant against *S. aureus*, *L. monocytogenes*, *K. pneumoniae,* and *E. coli* was recorded as 12.25 ± 0.31, 11.13 ± 0.12, 9.29 ± 0.41 and 10.25 ± 0.11 mm, respectively. At a concentration of 100 mg/mL, the zone of inhibition of the petroleum ether extract of *C. macrostachyus* leaf against *S. aureus*, *L. monocytogen*, *K. pneumoniae,* and *E. coli* was 9.79 ± 0.11, 85 ± 0.02, 9.61 ± 0.16 and 10.11 ± 0.62 mm, respectively.

Table 4. Zone of inhibitions measured for different concentrations of *C. macrostachyus* leaves extracts and isolated compounds against a bacterial pathogen.

Bacteria	Conc. (mg/mL)	Types of Extract			Isolated Compounds		DMSO	Chloramphenicol
		Pet-Ether	*$CHCl_3$*	*MeOH*	*Lupeol*	*Hardwickiic*		
E. coli	50	9.59 ± 0.21	11.05 ± 0.42	12.25 ± 0.31	6.03 ± 0.12	5.02 ± 0.43	0	9.45 ± 0.29
	100	10.10 ± 0.62	13.04 ± 0.11	13.75 ± 0.24	7.01 ± 0.32	5.85 ± 0.13		
	150	11.69 ± 0.45	14.11 ± 0.13	15.83 ± 0.22	7.92 ± 0.13	6.55 ± 0.21		
K. pneumoniae	50	9.08 ± 0.13	9.35 ± 0.28	11.13 ± 0.12	5.00 ± 0.11	5.67 ± 0.44	0	7.32 ± 0.15
	100	9.61 ± 0.16	10.53 ± 0.26	12.93 ± 0.32	5.90 ± 0.02	5.17 ± 0.33		
	150	11.27 ± 0.32	13.06 ± 0.09	14.17 ± 0.11	6.39 ± 0.24	6.50 ± 0.11		
S. aureus	50	9.09 ± 0.74	8.69 ± 0.25	9.29 ± 0.41	6.10 ± 0.21	5.02 ± 0.01	0	9.03 ± 0.49
	100	9.79 ± 0.11	9.59 ± 0.32	12.15 ± 0.23	6.99 ± 0.33	5.69 ± 0.14		
	150	11.18 ± 0.07	11.81 ± 0.12	13.75 ± 0.17	7.09 ± 0.36	6.01 ± 0.34		
L. monocytogenes	50	8.26 ± 0.06	9.18 ± 0.30	10.25 ± 0.11	5.89 ± 0.43	4.63 ± 0.21	0	8.07 ± 0.93
	100	8.85 ± 0.07	9.55 ± 0.28	11.55 ± 0.14	6.19 ± 0.55	5.12 ± 0.11		
	150	11.20 ± 0.45	11.81 ± 0.12	13.05 ± 0.21	6.97 ± 0.23	5.96 ± 0.41		

At a concentration of 100 mg/mL, the zone of inhibition of the chloroform extract of *C. macrostachyus* leaf against *S. aureus*, *L. monocytogenes*, *K. pneumoniae,* and *E. coli* was 9.59 ± 0. 32, 9.55 ± 0.28, 10.53 ± 0.26 and 13.04 ± 0.11 mm, respectively. Similarly, at the same concentration, the MeOH extracts against *S. aureus*, *L. monocytogenes*, *K. pneumoniae,* and *E. coli* were 13.75 ± 0.24, 12.93 ± 0.32, 12.15 ± 0.23 and 11.55 ± 0.14 mm, respectively. At a concentration of 150 mg/mL, pet ether extract inhibits *S. aureus*, *L. monocytogenes*, *K. pneumoniae,* and *E. coli* 11.18 ± 0.02, 11.02 ± 0.45, 11.27 ± 0.34, and 11.69 ± 0.45 mm, respectively. At a concentration of 150 mg/mL, the chloroform extracts inhibit *S. aureus*, *L. monocytogenes*, *K. pneumoniae,* and *E. coli,* 11.81 ± 0.12, 11.81 ± 0.12, 13.06 ± 0.09 and 14.11 ± 0.13 mm, respectively. In the same way, the MeOH extracts against the given pathogen inhibit 15.83 ± 0.22, 14.17 ± 0.11, 13.75 ± 0.17 and 13.05 ± 0.21 mm, respectively.

The plant extracts showed as low as 8.26 ± 0.06 mm to as high as 15.83 ± 0.22 mm diameter inhibition zones (Table 4). Petroleum ether extract has shown the least inhibition zones (8.26 ± 0.06 mm) against *L. monocytogenes*. MeOH extract has shown the highest inhibition zone against isolates of *E. coli* (15.83 ± 0.22 mm). For petroleum ether extracts, the highest inhibition zone (11.69 ± 0.45 mm) was recorded against *E. coli*, while the minimum inhibition was seen against *L. monocytogenes* (8.26 ± 0.06 mm). The maximum inhibition zone for chloroform extract was seen against *E. coli* (14.11 ± 0.13 mm), while the minimum inhibition was against *S. aureus* (8.69 ± 0.25 mm). Similarly, the maximum inhibition zone for MeOH extract was seen against *E. coli* (15.83 ± 0.22 mm), while the minimum inhibition was against *S. aureus* (9.29 ± 0.41 mm). In this study, except for the petroleum ether extract at 50 mg/mL concentration against *S. aureus* (8.69 ± 0.25 mm), all concentrations of the crude extracts showed higher inhibition than positive control against tested pathogens.

The antibacterial activity of pure isolated compounds was also evaluated against those given pathogens. Lupeol showed the maximum zone of inhibition against *E. coli* (7.92 ± 0.13

mm), whereas the minimum zone of inhibition against *K. pneumoniae* (5.00 ± 0.11 mm). Similarly, hardwickiic acid showed a maximum inhibition zone against E. coli (6.55 ± 0.21 mm) and a minimum inhibition zone against *L. monocytogenes* (4.63 ± 0.21 mm). The activity trend of pure compounds was almost similar to the extracts.

All crude extracts of the leaf of *C. macrostachyus* showed significant in vitro antibacterial activity against all tested bacterial strains at the concentrations of 50 mg/mL, 100 mg/mL, and 150 mg/mL. The antibacterial activity of extracts of *C. macrostachyus* against tested pathogens was due to the presence of secondary metabolites, such as flavonoids and alkaloids, which is in agreement with the results of the previous study [48]. However, crude extracts were recorded as more potent than pure compounds and positive control chloramphenicol toward the given pathogens because of the synergy effects of multiple compounds in the extracts, as shown in Table 5. The experimental results from this study were compared with the literature for crude extracts [58] and isolated compounds [59,60], which showed promising results.

Table 5. Maximum antibacterial activities of crude extracts and isolated compounds at 150 mg/ml.

Samples	Diameter of Zone of Growth Inhibition (mm) (Mean ± S.D.)			
	Bacterial Strains			
	E. coli	K. pneumoniae	S. aureus	L. monocytogenes
Pet-Ether	11.69 ± 0.45	11.27 ± 0.32	11.18 ± 0.07	11.20 ± 0.45 (
$CHCl_3$	14.11 ± 0.13	13.06 ± 0.09	11.81 ± 0.12	11.81 ± 0.12
MeOH	15.83 ± 0.22	14.17 ± 0.11	13.75 ± 0.17	13.05 ± 0.21
Lupeol	7.92 ± 0.13	6.39 ± 0.24	7.09 ± 0.36	6.97 ± 0.23
Hardwickiic acid	6.55 ± 0.21	6.50 ± 0.11	6.01 ± 0.34	5.96 ± 0.41
Chloramphenicol	9.45 ± 0.29 (15)	7.32 ± 0.15 (15)	9.03 ± 0.49 (15)	8.07 ± 0.93 (15)
DMSO	0	0	0	0

4. Conclusions

In this study, phytochemical analysis of methanol, chloroform, and petroleum ether extracts of C. macrostachyus leaves showed the presence of major secondary metabolites. Alka-loids, terpenoids, and saponins were found in all of the crude extracts, and anthraquinones were not detected in all extracts. The antibacterial activity of the tested plant revealed both extracts show higher inhibition than positive control against *E. coli*, *S. aureus*, *L. monocytogenes*, and *K. pneumoniae* in all concentrations except the 50 mg/mL concentration of petroleum ether extract against *S. aureus*. Strong activity was recorded by crude extracts against the selected bacteria strain when compared with isolated compounds. As reported in numerous studies, this is due to the synergy effect of different phytochemical constituents within the extract. The MeOH extract was the most effective antibacterial agent among all samples analyzed in this work. This has been ascribed to the presence of some active constituents in the polar extract of MeOH. The two isolated compounds, lupeol, and hardwickiic acid, also exhibited significant activity toward the given pathogens. The results obtained from this study indicated that *C. macrostachyus* has the potential to inhibit tested human pathogenic bacteria.

Funding

This research received no external funding.

Acknowledgments

This work was carried out at the Department of Chemistry and Biology, Wollo University, and Addis Ababa University, Ethiopia to facilitate the necessary materials, bioactivity test, and spectral analysis.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Pollastro, F.; Minassi, A. Exploring the Universe of Natural Products: Recent Advances in Synthesis, Isolation and Structural Elucidation. *Plants* **2021**, *10*, 2368, doi:10.3390/plants10112368.
2. Banerjee, P.; Mandhare, A.; Bagalkote, V. Marine natural products as source of new drugs: an updated patent review (July 2018-July 2021). *Expert Opinion on Therapeutic Patents* **2022**, *32*, 317-363, doi:10.1080/13543776.2022.2012150.
3. Atanasov, A.G.; Zotchev, S.B.; Dirsch, V.M.; Orhan, I.E.; Banach, M.; Rollinger, J.M.; Barreca, D.; Weckwerth, W.; Bauer, R.; Bayer, E.A.; et al. Natural products in drug discovery: advances and opportunities. *Nature Reviews Drug Discovery* **2021**, *20*, 200-216, doi:10.1038/s41573-020-00114-z.
4. Jayalakshmi, B.; Raveesha, K.A.; Amruthesh, K.N. Isolation and characterization of bioactive compounds from Euphorbia cotinifolia. *Future Journal of Pharmaceutical Sciences* **2021**, *7*, 9, doi:10.1186/s43094-020-00160-9.
5. Li, J.W.-H.; Vederas, J.C. Drug Discovery and Natural Products: End of an Era or an Endless Frontier? *Science* **2009**, *325*, 161-165, doi:doi:10.1126/science.1168243.
6. Seyedjavadi, S.S.; Razzaghi-Abyaneh, M.; Nasiri, M.J.; Hashemi, A.; Goudarzi, H.; Haghighi, M.; Dadashi, M.; Goudarzi, M.; Zare-Zardini, H.; Pourhossein, B. Isolation and Chemical Characterization of an Alpha-Helical Peptide, Dendrocin-ZM1, Derived from Zataria multiflora Boiss with Potent Antibacterial Activity. *Probiotics and antimicrobial proteins* **2022**, *14*, 326-336, doi:10.1007/s12602-022-09907-7.
7. Pacios, O.; Blasco, L.; Bleriot, I.; Fernandez-Garcia, L.; González Bardanca, M.; Ambroa, A.; López, M.; Bou, G.; Tomás, M. Strategies to Combat Multidrug-Resistant and Persistent Infectious Diseases. *Antibiotics* **2020**, *9*, 65-67, doi:10.3390/antibiotics9020065.
8. Afonso, A.C.; Sousa, M.; Simões, L.C.; Simões, M. Phytochemicals Against Drug-Resistant Bacterial Biofilms and Use of Green Extraction Solvents to Increase Their Bioactivity. *Advances in experimental medicine and biology* **2023**, *1370*, 1-18, doi:10.1007/5584_2022_723.
9. Jagessar, R.; Mars, A.; Gomes, G. Selective Antimicrobial properties of Phyllanthus acidus leaf extract against Candida albicans, Escherichia coli and Staphylococcus aureus using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method. *Nature and Science* **2008**, *6*, 24-38, doi:10.1007/s00210-023-02624-0.
10. Shetty, R.A. An Overview on Medicinal Plants with Potential Antidepressant Activity. *Int. J. Life Sci. Pharma Res 10*, 180-185, doi:10.22376/ijpbs/lpr.2020.10.5.L180-185.
11. Asefa, M.; Cao, M.; He, Y.; Mekonnen, E.; Song, X.; Yang, J. Ethiopian vegetation types, climate and topography. *Plant Diversity* **2020**, *42*, 302-311, doi:10.1016/j.pld.2020.04.004.
12. Getachew, S.; Medhin, G.; Asres, A. Traditional medicinal plants used in the treatment of tuberculosis in Ethiopia: A systematic review. *Heliyon* **2022**, *8*, 1-12, doi:10.1016/j.heliyon.2022.e09478.

13. Lutoti, S.; Kaggwa, B.; Kamba, P.F.; Mukonzo, J.; Sesaazi, C.D.; Katuura, E. Ethnobotanical Survey of Medicinal Plants Used in Breast Cancer Treatment by Traditional Health Practitioners in Central Uganda. *Journal of multidisciplinary healthcare* **2023**, *16*, 635-651, doi:10.2147/jmdh.s387256.
14. Espinoza-Hernández, F.A.; Moreno-Vargas, A.D.; Andrade-Cetto, A. Diabetes-Related Mechanisms of Action Involved in the Therapeutic Effect of Croton Species: A Systematic Review. *Plants (Basel, Switzerland)* **2023**, *12*, 1-7, doi:10.3390/plants12102014.
15. Bekono, B.D.; Ntie-Kang, F.; Onguéné, P.A.; Lifongo, L.L.; Sippl, W.; Fester, K.; Owono, L.C. The potential of anti-malarial compounds derived from African medicinal plants: a review of pharmacological evaluations from 2013 to 2019. *Malaria Journal* **2020**, *19*, 1-35, doi:10.1186/s12936-020-03231-7.
16. Weniger, B.; Lagnika, L.; Vonthron-Sénécheau, C.; Adjobimey, T.; Gbenou, J.; Moudachirou, M.; Brun, R.; Anton, R.; Sanni, A. Evaluation of ethnobotanically selected Benin medicinal plants for their in vitro antiplasmodial activity. *Journal of Ethnopharmacology* **2004**, *90*, 279-284, doi:10.1016/j.jep.2003.10.002.
17. Beressa, T.B.; Deyno, S.; Mtewa, A.G.; Aidah, N.; Tuyiringire, N.; Lukubye, B.; Weisheit, A.; Tolo, C.U.; Ogwang, P.E. Potential Benefits of Antiviral African Medicinal Plants in the Management of Viral Infections: Systematic Review. *Front Pharmacol* **2021**, *12*, 1-13, doi:10.3389/fphar.2021.682794.
18. Osman, A.; Sbhatu, D.B.; Giday, M. Medicinal Plants Used to Manage Human and Livestock Ailments in Raya Kobo District of Amhara Regional State, Ethiopia. *Evid Based Complement Alternat Med* **2020**, *2020*, 1-19, doi:10.1155/2020/1329170.
19. Constantinoiu, C.; Croton, C.; Paterson, M.B.A.; Knott, L.; Henning, J.; Mallyon, J.; Coleman, G.T. Prevalence of canine heartworm infection in Queensland, Australia: comparison of diagnostic methods and investigation of factors associated with reduction in antigen detection. *Parasit Vectors* **2023**, *16*, 63-66, doi:10.1186/s13071-022-05633-9.
20. Abebe, W. A survey of prescriptions used in traditional medicine in Gondar region, northwestern Ethiopia: general pharmaceutical practice. *Journal of ethnopharmacology* **1986**, *18*, 147-165, doi:10.1016/0378-8741(86)90027-9.
21. Mazzanti, G.; Bolle, P.; Martinoli, L.; Piccinelli, D.; Grgurina, I.; Animati, F.; Mugnè, Y. Croton macrostachys, a plant used in traditional medicine: purgative and inflammatory activity. *Journal of ethnopharmacology* **1987**, *19*, 213-219, doi:10.1016/0378-8741(87)90043-2.
22. Filippidis, P.; Francini, K.; Jacot-Guillarmod, M.; Mathevet, P.; Lhopitallier, L.; Cavassini, M.; Darling, K.E.A. HIV testing in termination of pregnancy and colposcopy services: a scoping review. *Sexually transmitted infections* **2022**, *98*, 143-149, doi:10.1136/sextrans-2021-055111.
23. Prasad, S.; Yadav, V.R.; Sundaram, C.; Reuter, S.; Hema, P.S.; Nair, M.S.; Chaturvedi, M.M.; Aggarwal, B.B. Crotepoxide chemosensitizes tumor cells through inhibition of expression of proliferation, invasion, and angiogenic proteins linked to proinflammatory pathway. *Journal of Biological Chemistry* **2010**, *285*, 26987-27000, doi:10.1074/jbc.A110.121061.
24. Luu-Dam, N.A.; Le, C.V.C.; Satyal, P.; Le, T.M.H.; Bui, V.H.; Vo, V.H.; Ngo, G.H.; Bui, T.C.; Nguyen, H.H.; Setzer, W.N. Chemistry and Bioactivity of Croton Essential Oils: Literature Survey and Croton hirtus from Vietnam. *Molecules* **2023**, *28*, 1-39, doi:10.3390/molecules28052361.
25. Belay, D.W.; Asfaw, Z.; Lulekal, E.; Kassa, B.; Kifele, H. Effects of essential oils on potato tuber sprouting at room temperature storage in Ethiopia. *Heliyon* **2022**, *8*, 1-6, doi:10.1016/j.heliyon.2022.e09090.

26. Ayele, D.T.; Akele, M.L.; Melese, A.T. Analysis of total phenolic contents, flavonoids, antioxidant and antibacterial activities of Croton macrostachyus root extracts. *BMC Chem.* **2022**, *16*, 30-35, doi:10.1186/s13065-022-00822-0.
27. Wondafrash, M. A preliminary guide to plant collection, identification and herbarium techniques. *Addis Ababa. The national Herbarium AAU* **2008**, p1-58.
28. Devkota, A.; Das, R.K. Phytochemical screening and in-vitro evaluation of antimicrobial activity of invasive species Ageratina adenophora collected from Kathmandu valley, Nepal. *Scientific World* **2022**, *15*, 120-126, doi:10.3126/sw.v15i15.45660.
29. Mazzara, E.; Torresi, J.; Fico, G.; Papini, A.; Kulbaka, N.; Dall'Acqua, S.; Sut, S.; Garzoli, S.; Mustafa, A.M.; Cappellacci, L. A Comprehensive Phytochemical Analysis of Terpenes, Polyphenols and Cannabinoids, and Micromorphological Characterization of 9 Commercial Varieties of Cannabis sativa L. *Plants* **2022**, *11*, 891-922, doi:10.3390/plants11070891.
30. Dubale, S.; Kebebe, D.; Zeynudin, A.; Abdissa, N.; Suleman, S. Phytochemical Screening and Antimicrobial Activity Evaluation of Selected Medicinal Plants in Ethiopia. *Journal of experimental pharmacology* **2023**, *15*, 51-62, doi:10.2147/jep.s379805.
31. Anusmitha, K.M.; Aruna, M.; Job, J.T.; Narayanankutty, A.; Benil, P.; Rajagopal, R.; Alfarhan, A.; Barcelo, D. Phytochemical analysis, antioxidant, anti-inflammatory, anti-genotoxic, and anticancer activities of different Ocimum plant extracts prepared by ultrasound-assisted method. *Physiological and Molecular Plant Pathology* **2022**, *117*, 101746-101758, doi:/10.1016/j.pmpp.2021.101746.
32. Chiangnoon, R.; Samee, W.; Uttayarat, P.; Jittachai, W.; Ruksiriwanich, W.; Sommano, S.R.; Athikomkulchai, S.; Chittasupho, C. Phytochemical Analysis, Antioxidant, and Wound Healing Activity of Pluchea indica L.(Less) Branch Extract Nanoparticles. *Molecules* **2022**, *27*, 635-655, doi:10.3390/molecules27030635.
33. Farooq, S.; Shaheen, G.; Asif, H.M.; Aslam, M.R.; Zahid, R.; Rajpoot, S.R.; Jabbar, S.; Zafar, F. Preliminary Phytochemical Analysis: In-Vitro Comparative Evaluation of Anti-arthritic and Anti-inflammatory Potential of Some Traditionally Used Medicinal Plants. *Dose-Response* **2022**, *20*, 1-8, doi:10.1177/15593258211069720.
34. Chen, C.; Zhu, H.; Kang, J.; Warusawitharana, H.K.; Chen, S.; Wang, K.; Yu, F.; Wu, Y.; He, P.; Tu, Y. Comparative Transcriptome and Phytochemical Analysis Provides Insight into Triterpene Saponin Biosynthesis in Seeds and Flowers of the Tea Plant (Camellia sinensis). *Metabolites* **2022**, *12*, 204-218, doi:10.3390/metabo12030204.
35. Masi, M.; Evidente, A. Fungal bioactive anthraquinones and analogues. *Toxins* **2020**, *12*, 714-719, doi:10.3390/toxins12110714.
36. Qiu, Z.; Li, C.-J. Transformations of less-activated phenols and phenol derivatives via C–O cleavage. *Chemical Reviews* **2020**, *120*, 10454-10515, doi:10.1021/acs.chemrev.0c00088.
37. Xu, L.-Y.; Fan, N.-L.; Hu, X.-G. Recent development in the synthesis of C-glycosides involving glycosyl radicals. *Organic & Biomolecular Chemistry* **2020**, *18*, 5095-5109, doi:10.1039/D0OB00711K.
38. Saleem, B.; Islam, M.; Ahmed, A.; Saeed, H.; Imtiaz, F.; Muzaffar, S. Bioassay-guided isolation and in silico study of antihyperlipidemic compounds from Onosma hispidum Wall. *Journal of biomolecular structure & dynamics* **2023**, 1-15, doi:10.1080/07391102.2023.2242503.
39. Zhang, J.; Chu, W.C.; Li, L.Z. Isolation and structure elucidation of antioxidant compounds from stem and root barks of Daphne giraldii. *J Asian Nat Prod Res* **2023**, 1-10, doi:10.1080/10286020.2023.2195106.
40. Andriani, D.; Reviantti, S.; Prananingrum, W. Identification of compounds isolated from a methanolic extract of Acanthus ilicifolius leaves and evaluation of their

antifungal and antioxidant activity. *Biodiversitas Journal of Biological Diversity* **2020**, *21*, 1-13, doi:10.13057/biodiv/d210625.
41. Bhuyar, P.; Rahim, M.H.A.; Maniam, G.P.; Ramaraj, R.; Govindan, N. Exploration of bioactive compounds and antibacterial activity of marine blue-green microalgae (Oscillatoria sp.) isolated from coastal region of west Malaysia. *SN Applied Sciences* **2020**, *2*, 1-10.
42. Jobaer, M.A.; Ashrafi, S.; Ahsan, M.; Hasan, C.M.; Rashid, M.A.; Islam, S.N.; Masud, M.M. Phytochemical and Biological Investigation of an Indigenous Plant of Bangladesh, Gynura procumbens (Lour.) Merr.: Drug Discovery from Nature. *Molecules* **2023**, *28*, 1-16, doi:10.3390/molecules28104186.
43. Angelini, P.; Venanzoni, R.; Angeles Flores, G.; Tirillini, B.; Orlando, G.; Recinella, L.; Chiavaroli, A.; Brunetti, L.; Leone, S.; Di Simone, S.C. Evaluation of antioxidant, antimicrobial and tyrosinase inhibitory activities of extracts from Tricholosporum goniospermum, an edible wild mushroom. *Antibiotics* **2020**, *9*, 513-521, doi:10.3390/antibiotics9080513.
44. Hirano, R.; Nishita, I.; Nakai, R.; Bito, A.; Sasabe, R.; Kurihara, S. Development of culture methods capable of culturing a wide range of predominant species of intestinal bacteria. *Frontiers in cellular and infection microbiology* **2023**, *13*, 1-15, doi:10.3389/fcimb.2023.1056866.
45. Anzano, A.; de Falco, B.; Ammar, M.; Ricciardelli, A.; Grauso, L.; Sabbah, M.; Capparelli, R.; Lanzotti, V. Chemical Analysis and Antimicrobial Activity of Moringa oleifera Lam. Leaves and Seeds. *Molecules* **2022**, *27*, 1-12, doi:10.3390/molecules27248920.
46. Saddiq, A.A.; Tag, H.M.; Doleib, N.M.; Salman, A.S.; Hagagy, N. Antimicrobial, Antigenotoxicity, and Characterization of Calotropis procera and Its Rhizosphere-Inhabiting Actinobacteria: In Vitro and In Vivo Studies. *Molecules* **2022**, *27*, 1-19, doi:10.3390/molecules27103123.
47. Fajt, V.R.; Lubbers, B.V. Application and Interpretation of Antimicrobial Susceptibility Testing. *The Veterinary clinics of North America. Food animal practice* **2023**, *39*, 115-128, doi:10.1016/j.cvfa.2022.09.001.
48. Obey, J.K.; von Wright, A.; Orjala, J.; Kauhanen, J.; Tikkanen-Kaukanen, C. Antimicrobial activity of Croton macrostachyus stem bark extracts against several human pathogenic bacteria. *Journal of Pathogens* **2016**, *2016(1453428)*, 1-16, doi:10.1155/2016/1453428.
49. Morais, L.V.F.d.; Luz, J.R.D.d.; Nascimento, T.E.S.d.; Azevedo, M.A.d.S.; Rocha, W.P.d.S.; Araujo-Silva, G.; Ururahy, M.A.G.; Chaves, G.M.; Brandão-Neto, J.; López, J.A. Phenolic composition, toxicity potential, and antimicrobial activity of licania rigida benth (chrysobalanaceae) leaf extracts. *Journal of Medicinal Food* **2022**, *25*, 97-109, doi:10.1089/jmf.2021.0059.
50. de Carvalho, T.C.; Polizeli, A.M.; Turatti, I.C.; Severiano, M.E.; de Carvalho, C.E.; Ambrósio, S.R.; Crotti, A.E.; de Figueiredo, U.S.; Vieira, P.C.; Furtado, N.A. Screening of filamentous fungi to identify biocatalysts for lupeol biotransformation. *Molecules* **2010**, *15*, 6140-6151, doi:10.3390/molecules15096140.
51. Ahmad, A.; Siddiqui, P.J.A.; Fayyaz, S.; Khan, K.; Iqbal, E.Y.; Rasheed, M.; Muzafar, W.; Faizi, S. Bioassay Directed Fractionation of Petroleum Ether Extract of Aerial Parts of Ceriops tagal: Isolation of Lupeol as the Nematicidal Agent against Cyst Nematode Heterodera zeae. *Chemistry & Biodiversity* **2022**, *19*, 1-11, doi:10.1002/cbdv.202100759.
52. Merritt, A.; Ley, S. Clerodane diterpenoids. *Natural Product Reports* **1992**, *9*, 243-287, doi:10.1039/NP9920900243.

53. Li, R.; Morris-Natschke, S.L.; Lee, K.-H. Clerodane diterpenes: sources, structures, and biological activities. *Natural product reports* **2016**, *33*, 1166-1226, doi:10.1039/c5np00137d.
54. Cai, Y.; Chen, Z.P.; Phillipson, J.D. Diterpenes from Croton lechleri. *Phytochemistry* **1993**, *32*, 755-760, doi:https://doi.org/10.1016/S0031-9422(00)95166-5.
55. Ledoux, A.; Hamann, C.; Bonnet, O.; Jullien, K.; Quetin-Leclercq, J.; Tchinda, A.; Smadja, J.; Gauvin-Bialecki, A.; Maquoi, E.; Frédérich, M. Bioactive Clerodane Diterpenoids from the Leaves of Casearia coriacea Vent. *Molecules* **2023**, *28 (3)1197*, 1-10, doi:10.3390/molecules28031197.
56. Abdullahi, S.; Musa, A.; Abdullahi, M.; Sule, M.; Sani, Y. Isolation of Lupeol from the Stem-bark of Lonchocarpus sericeus (Papilionaceae). *Scholars Academic Journal of Biosciences* **2013**, *1*, 18-19, doi:10.36347/sajb.2013.v01i01.004
57. Santos, A.G.d.; Perez, C.C.; Tininis, A.G.; Bolzani, V.d.S.; Cavalheiro, A.J. Clerodane diterpenes from leaves of Casearia sylvestris Swartz. *Química Nova* **2007**, *30*, 1100-1103, doi:10.1590/S0100-40422007000500009.
58. Kibret, B.; Alemayehu, I.; Endale, M.; Asegu, D. Comparative Study of the Antibacterial Activity of Leaves of Croton Macrostachyus and Aloe Vera. **2018**.
59. Vieira, R.G.L.; Moraes, T.d.S.; Silva, L.d.O.; Bianchi, T.C.; Veneziani, R.C.S.; Ambrósio, S.R.; Bastos, J.K.; Pires, R.H.; Martins, C.H.G. In vitro studies of the antibacterial activity of Copaifera spp. oleoresins, sodium hypochlorite, and peracetic acid against clinical and environmental isolates recovered from a hemodialysis unit. *Antimicrobial Resistance & Infection Control* **2018**, *7(14)*, 1-13, doi:10.1186/s13756-018-0307-3.
60. Akwu, N.; Naidoo, Y.; Singh, M.; Channangihalli Thimmegowda, S.; Nundkumar, N.; Lin, J. Isolation of lupeol from Grewia lasiocarpa E.Mey. ex Harv. stem bark: Antibacterial, antioxidant and cytotoxicity activities. *Biodiversitas Journal of Biological Diversity* **2020**, *21*, 5684-5690, doi:10.13057/biodiv/d211213.