



Preliminary Phytochemical Screening, Isolation, Characterization, Structural Elucidation and Antibacterial Activities of Leaves Extracts *Rhus vulgaris* (Kimmo)

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Abstract

Rhus vulgaris, commonly known as sumac, is a plant known for its various therapeutic properties, including antioxidant and antibacterial activities. Medicines derived from plants significantly contribute to human health. This study aimed to screen the phytochemical constituents, isolate and elucidate the structure, and evaluate the antibacterial activity of the methanol extract from the leaves of *Rhus vulgaris*. The concentrated fraction was purified using thin-layer chromatography (TLC) and column chromatography to isolate the pure compounds. The isolated compounds were characterized using Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR). Antibacterial activity was tested against four bacterial strains: *Streptococcus aureus* (gram-positive), *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* (gram-negative) using the agar well diffusion method. The methanol extract exhibited antibacterial activity against all tested bacteria, with significant inhibition zones, particularly against *Streptococcus aureus* (15 mm) and *Salmonella typhimurium* (14 mm). The preliminary phytochemical screening of the extracts revealed the presence of alkaloids, glycosides, steroids, anthraquinones, and carbohydrates were detected in all extracts. The methanolic extract of *Rhus vulgaris* was subjected to column chromatography and eluted with methanol: chloroform (1:8) mixture, leading to the identification of the compound 1-p-tolyl pentadeca-7, 9-dien-1-ol. The methanolic extract of *Rhus vulgaris* has demonstrated strong antibacterial activity, indicating its potential as an effective antimicrobial agent.



Article History

Received: 06 June 2024
Accepted: 27 July 2024

Keywords:

Antibacterial Activities;
Isolation;
Phytochemical
Screening;
Rhus Vulgaris
Structural Elucidation.

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Doi: DOI : <http://dx.doi.org/10.13005/OJPS09.02.08>

Introduction

Natural products, which contribute nearly 50% of new chemical entities in drug development, are crucial in providing starting points for synthetic drugs.¹ Medicinal plants have been used since ancient times for various purposes and continue to guide the search for new medications due to their accessibility, affordability, and minimal side effects compared to synthetic drugs. These plants contain phytochemicals that produce specific physiological actions.² The medicinal efficacy of plants is attributed to these chemical substances, known as phytochemicals, which exert definite physiological effects on the human body.^{3,4}

The *Rhus* genus, known as sumac, comprises over 250 flowering plant species in the Anacardiaceae family,⁵ predominantly found in tropical, subtropical, and temperate regions worldwide. The name "sumac" originated from the Syriac word "sumaga," meaning red. These non-agricultural plants have been utilized by indigenous people for medicinal and other purposes, indicating their potential for commercial application without competing with food production.⁶ Traditional *Rhus* species have been employed to treat various ailments, including influenza, wounds, diarrhea, stomach pain, indigestion, diabetes, malaria, rheumatism, gum and toothaches, swollen legs, dog bites, peptic ulcers, kidney stones, skin eruptions, bruises, and boils.^{7,8} In Ethiopia, *R. vulgaris* is used to manage various diseases according to ethnobotanical and ethno-pharmacological research, such as diarrhea, gonorrhoea, infections, the evil eye, wounds, and lung tuberculosis in the Amhara region.^{9,10} In Kenya, this plant's stem bark is used to treat malaria.¹¹ In Tanzania, Fresh leaves are burned and the ash is used for oxytocic action, and externally, the ash is applied for the treatment of scabies. The plant is used to stop diarrhea, wounds, gonorrhoea, and infertility, and to ease delivery.¹² In Uganda, this plant is used to treat diarrhea, malaria, hemorrhoids,¹³ Yellow fever, Cough, Malaria, Gastro-intestinal disorders, toothaches, Syphilis, Immunity booster, Smallpox, Swollen lymph, and Prevention diseases,¹⁴ more preferable chewing sticks over synthetic toothbrushes.¹⁵ In Kenya, the roots, leaves, and fruits are used as the treatment of cancers like Stomach, skin, and breast cancer.¹⁶

In recent studies, researchers have successfully isolated and characterized novel compounds from *Rhus* species, demonstrating their therapeutic potential. These findings underscore the importance of further exploring *Rhus vulgaris* for its pharmacological benefits and developing these natural products into clinically valuable drugs. Phytochemical studies on *Rhus vulgaris* stem bark have identified tannins, saponins, flavonoids, terpenoids, glycosides, alkaloids, and phenols. The methanolic extract showed superior anti-inflammatory activity compared to indomethacin.¹⁷ Other studies have identified new biflavonoids in *Rhus* species, including agathisflavone, amentoflavone, hinokiflavone, rhus flavanone, and succedanea flavone, which exhibit activity against various significant viruses. Hinokiflavone emerged as the most active among 65 natural flavonoids in inhibiting the pro-coagulant activity of adherent human monocytes stimulated by endotoxin and interleukin-1- β in vitro. Additionally, other *Rhus* biflavonoids demonstrate cytotoxic and antimalarial activities.^{18,19}

This study aims to isolate potentially bioactive compounds from *Rhus vulgaris* leaves using column chromatography and preparative thin-layer chromatography. Structural elucidation of the active compound will be conducted using spectroscopic techniques such as FTIR and NMR. Furthermore, the study seeks to assess the bioactivity of *Rhus vulgaris* leaves against various pathogens.

Description

Rhus vulgaris is found in all parts of Tanzania; Uganda and Kenya and from Cameroon to Ethiopia and south to Mozambique, Malawi, Zambia, and Zimbabwe.²⁰ It is a shrub or small tree that occasionally reaches 1-9 m; its bark is smooth, dark brown, its branches yellow-red-brown, and often densely hairy. Leaves are 3 leaflets, dull green, softly hairy, the central leaflet larger, 4.11 cm long x 2.6.5 cm wide, the two laterals smaller, shortly stalked, edge entire or soft toothed towards the tip, which is blunt or pointed, leaflets dark above, paler below. Flowers are small cream-green-yellow, parts in fives, in terminal loose heads or from upper leaf axils, 5.20 cm long, all densely hairy. Fruits are drupes, with thin flesh, flat and round, red-brown, and only 3.5 mm across.²¹

Taxonomic Classification

According to the International Code of Botanical Nomenclature, the present taxonomic classification of *Rhus vulgaris* is shown in Figure 1

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Sapindales
Family	Anacardiaceae
Genus	<i>Rhus</i>
Species	<i>Rhus vulgaris</i> Meikle

Synonyms

Searsia pyroide (Burch.) Moffett
Rhus pyroide (Burch.)¹⁰

Material and Methods

Reagents and Chemicals

All the chemicals and reagents used in the present study were of analytical grade. The chemicals and reagents included: Methanol (98%), acetic anhydride, ferric chloride solution, ammonia, Mayer's reagent, Benedict's reagent, Sodium bicarbonate, ammonia, Ninhydrin, Molisch's reagent, Millon's reagent, lead acetate, Folin-Ciocalteu reagent, gallic acid, ascorbic acid, sodium hydroxide, anhydrous sodium carbonate, and anhydrous aluminum chloride, which were purchased from Loba Chemie (Mumbai, India); whereas sulfuric acid, acetic acid, ethanol, n-hexane, chloroform, acetone, ethyl acetate, DMSO, Vanillin solution (15g of vanillin in 250 ml of ethanol and add 2.5 ml of conc. H₂SO₄), NaOH, concentrated hydrochloric acid, silica gel (60-200 Mesh size) from (Oxford Lab. Chem., India), distilled water, are more relevant to make this experiment.

Apparatus and Instruments

The apparatus and instruments that were used for the study include: electrical grinder (IAK-WERKE, Germany), electronic balance (Bosch, Germany), Whatman No. 42 filter paper (110 mm), conical flasks, volumetric flasks, TLC plates, measuring cylinders, graduated pipettes, micropipettes (Mumbai, India), refrigerator (Hitachi LR902T, USA), orbital shaker (GEMMY Orbital Shaker, VRN-480, Taiwan), Autoclave, balance (Sartorius, Germany),

rotary evaporator (Bibby RE200, Sterilin Ltd., UK) and UV/Vis spectrophotometer (Sanyo SP75, UK) were used in the study.

FTIR

The isolate was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 400- 4000 cm⁻¹ in FT-IR spectroscopy (Bruker FTIR Spectrometer, USA).

Nuclear Magnetic Resonance Spectroscopy

The ¹H, ¹³C-NMR, and 1D NMR spectra of base degradation impurities were recorded in DMSO-d₆ solvent on Bruker 400 MHz Avance -III HD NMR spectrometer equipped with broadband observe (BBO) probe. The ¹H and ¹³C chemical shifts are reported on the δ scale in ppm, relative to tetramethyl silane (TMS) as an internal standard. The spectra were set to δ 0.00 ppm in ¹H NMR (TMS) and δ 39.50 ppm in ¹³C NMR (DMSO-d₆).

Collection of Plants

The fresh and healthy leaves of *R. vulgaris* were collected from a local farm located in the Region of Amara, specifically in Takusa Woreda Kebele 12, Central Gondar Zone, in February 2020. Taxonomic identification of the plant was conducted by botanist Mr. Getenet Chekol (MSc.) at the Department of Biology, University of Gondar.

After collection, the plant materials underwent thorough washing with tap water to eliminate any dust particles adhering to the leaves. Subsequently, the collected plant material was air-dried in the shade and stored at room temperature for 10 days. The dried leaves, which were in good condition, were then ground into a uniform powder using an electric grinder. The powdered samples were accurately weighed and stored in sealed containers for subsequent extraction purposes.

Extraction

The organic compounds present in *Rhus vulgaris* leaves were extracted and isolated using the maceration method, chosen for its ability to thoroughly extract the leaves without risking the thermal decomposition of any heat-sensitive compounds.²² The powdered leaves (800g) underwent sequential maceration with solvents of increasing polarity: n-hexane (C₆H₁₄) 2L, chloroform (CHCl₃) 2L, and

methanol (CH₃OH) 2 L, each for approximately 72 hours. Regular shaking ensured complete extraction as shown in Figure 2. The resulting extracts were decanted, filtered with Whatman filter paper, and concentrated using a rotary evaporator at temperatures specific to each solvent (n-hexane: 69°, chloroform: 61°, methanol: 65°). The crude extracts were then weighed using a digital balance, yielding

n-hexane (4.35 g), chloroform (14.7 g), and methanol (100.7g) extracts. Among these, the methanol extract exhibited superior antibacterial activity and underwent further purification via column chromatography, using a mixture of methanol: chloroform (1:8) determined through TLC trials to isolate the major component, as detailed in Table 1.



Fig. 1: The photograph of Rhus vulgaris Leaves.

Column Chromatographic

The methanol extract underwent silica gel (60-120 mesh ASTM, Merck) glass column chromatography with a diameter of 20-25 mm. Initially, silica gel (150 g) was mixed with chloroform to form a homogeneous slurry, which was then poured into the glass column

after stirring to remove bubbles. For sample loading, 6g of the extract was dissolved in 40 ml of methanol. To this solution, 10 g of silica was added and mixed thoroughly by stirring with a glass rod, followed by drying at room temperature. The resulting dried silica-extract mixture was layered onto the column bed.

Table 1: Solvent system used to separate AA1

No-	Solvent	Solvent Ratio	No. fractions	Code of Fraction	Rf value
1	MeOH: CHCl ₃	1:8	1-3	A ₁	0.9
2	MeOH: CHCl ₃	1:8	4-16	A ₂	0.85
3	MeOH: CHCl ₃	1:8	17-45	A ₃	0.70
4	MeOH: CHCl ₃	1:8	46-56	A ₄	0.75
5	MeOH: CHCl ₃	1:8	57-59	A ₅	0.8

The column was first eluted with a mobile phase consisting of methanol: chloroform in a 1:8 ratio and allowed to run until a consistent flow was achieved. A total of 59 fractions were collected during elution in Table 1. The profiles of column fractions were monitored by TLC to ensure similarity based on the number and appearance of spots on the plate, visualized using a UV lamp and vanillin solution.

Subsequently, the eluted compound was crystallized and subjected to Nuclear Magnetic Resonance (NMR) analysis (1H-NMR, 13C-NMR, DEPT-135) and Fourier transform infrared (FTIR) spectroscopy to elucidate its structure. The compound isolated from this fraction of column eluate was observed to be dark green and gummy in appearance.

Preliminary Phytochemical Screening

Phytochemical screening was conducted to evaluate the qualitative chemical composition of crude extracts, employing standard precipitation and coloration methods. This screening aimed to identify major natural chemical groups such as alkaloids, phenolic compounds, glycosides, carbohydrates, flavonoids, saponins, terpenoids, anthraquinones, tannins, steroids, amino acids, coumarins, and proteins. Through these analyses, the presence or absence of these compounds in the tested crude extracts was determined. The crude extracts, previously prepared and stored in a refrigerator, were utilized for these phytochemical tests.²³⁻²⁷

Antibacterial Activities of the Leaves Extracts of *Rhus vulgaris*

To assess the antimicrobial activity of the crude extract, the following test organisms were employed: *Streptococcus aureus* (gram-positive),

Escherichia coli, *Salmonella typhimurium*, and *Klebsiella pneumoniae* (gram-negative) bacteria. The antibacterial activity of both crude and purified extracts was determined using an agar well diffusion assay.

Mueller Hinton Agar (MHA) was utilized as the growth medium. Preparation involved dissolving 19 g of media powder in 500 ml of distilled water, followed by autoclaving at 121°C for 15 minutes to ensure sterilization. The cooled media was poured into plates, allowed to solidify, and positioned upright in the incubator to prevent contamination.

The bacterial cultures were grown on MHA, and inoculums were prepared for the antibacterial assay. Fresh cultures were picked from overnight growth, suspended in 3-4 ml of physiological saline in sterile test tubes, and adjusted for turbidity to match a 0.5 McFarland standard, corresponding to a bacterial load of approximately 1×10^8 CFU.

Extraction, Isolation and Purification

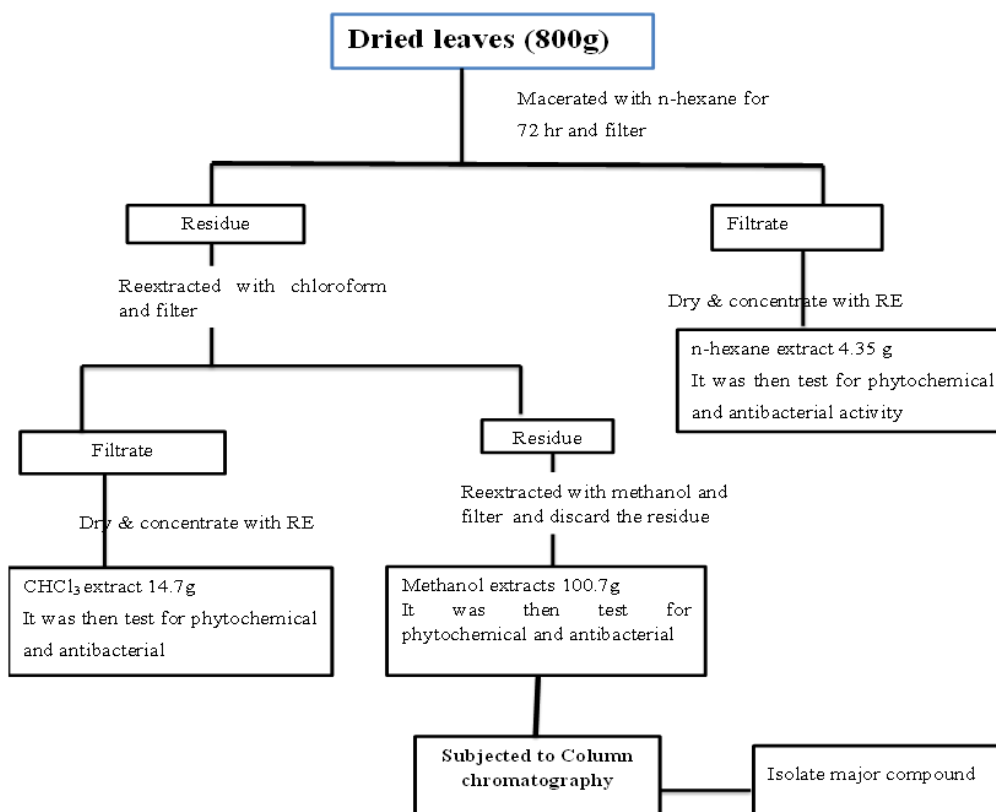


Fig. 2: Method of extraction of the plant material

Antibacterial activities of *Rhus vulgaris* extracts were evaluated against the four bacterial strains using the agar well diffusion method. The test bacteria were swabbed onto the surface of the leveled media and allowed to dry for 10 minutes. Wells were then bored into the plates using a sterilized well borer (6 mm diameter), and 100 mg/ml of each extract (n-hexane, chloroform, and methanol) dissolved in DMSO was added. Gentamicin discs (30 mcg/disc) served as the positive antibiotic control. Petri dishes were incubated at 37°C for 24 hours. After incubation, the zones of inhibition were measured and the average inhibition zone diameter was recorded in millimeters.

Results and Discussion

The Yield of Solvent Extract and Isolation of Leaves of *Rhus vulgaris*

The dried and powdered roots (800 g) of *Rhus vulgaris* underwent exhaustive extraction, successively employing n-hexane, chloroform, and methanol. The solvent from each extract was recovered under reduced pressure using a rotary evaporator, resulting in n-hexane (4.35 g), chloroform (14.7 g), and methanol (100.7 g) extracts as shown in Table 2. Chromatographic purification of the methanol

extract (7 g) yielded a compound coded as AA1. The structure of this compound has been elucidated based on spectroscopic evidence, as described in the following section.

Table 2: Percentage Yields of the Extracts.

Extracts	Weight	Yield (%)
n-hexane	4.35g	0.54
Chloroform	14.7g	1.84
Methanol	100.7g	12.6

Phytochemical Screening of the Leaves Extracts of *Rhus Vulgaris*

Phytochemical analysis of each crude extract from *Rhus vulgaris* indicated the presence of various pharmacologically significant secondary metabolite classes. Alkaloids, glycosides, steroids, anthraquinones, and carbohydrates were detected in all extracts. Phenols, flavonoids, tannins, coumarins, and proteins were exclusively found in methanol extracts, while terpenoids, saponins, and amino acids were absent in all extracts, as summarized in Table 3.

Table 3: Phytochemical constituents of the leaves extract of *Rhus vulgaris*.

Phytochemical constituent	n-hexane	Chloroform	Methanol
Alkaloids	+	+	++
Phenols	-	-	++
Glycosides	+	+	++
Flavonoids	-	-	++
Terpenoids	-	-	-
Steroids	+	+	+
Carbohydrates	+	+	+
Anthraquinones	+	+	+
Proteins	-	-	+
Coumarins	-	-	+
Tannins	-	-	+
Saponins	-	-	-
Amino acid	-	-	-

Key: (+) = Present, (-) = Absent

Antibacterial Activity of the Crude Extracts Against Bacterial Strain

The antibacterial activity of *Rhus vulgaris* extracts using different solvents was evaluated against

selected pathogens (Table 4). The methanol extract exhibited robust activity, showing inhibition zones of 15 mm against *Staphylococcus aureus*, 14 mm against *Salmonella typhi*, 11 mm against *Klebsiella*

pneumoniae, and 13 mm against *Escherichia coli*. In contrast, n-hexane and chloroform extracts showed limited effectiveness, with n-hexane displaying 3 mm against *S. aureus* and chloroform exhibiting 7 mm against both *S. aureus* and *E. coli* in Figure 3 and Figure 4.

These results underscore the potent antibacterial potential of the methanol extract from *Rhus vulgaris* leaves, particularly effective against *S. aureus*. However, *S. typhi* and *K. pneumoniae* exhibited partial resistance to the extracts.

Table 4: Antibacterial efficacy of extracts against pathogens

Extract	Inhibition zone(mm) against			
	<i>S. aureus</i>	<i>S. typhi</i>	<i>K. pneum</i>	<i>E. coli</i>
n-hexane	3	0	0	2
CHCl ₃	7	0	0	7
MeOH	15	14	11	13
Ge	18	17	14	15
DMSO	0	0	0	0

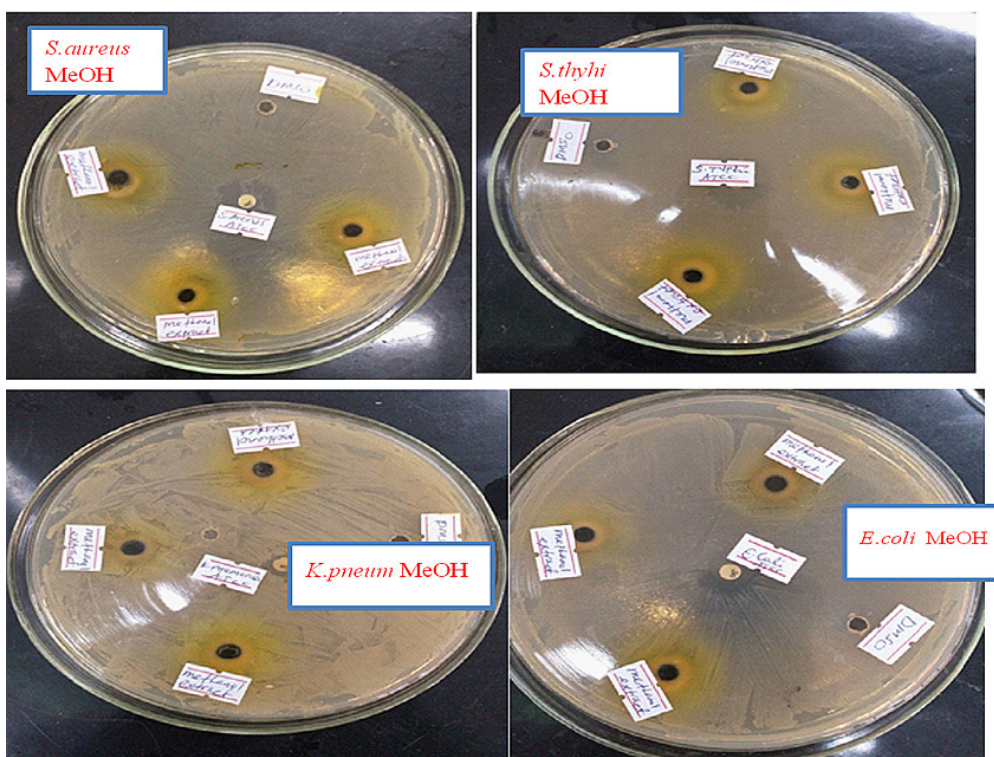


Fig. 3: Zone of inhibition of MeOH compared with an inhibition zone of standard antibiotic Gentamicin on *S.aureus*, *S.typhi*, *K. pneumonia*, and *E.coli*.

The methanol extract's superior antibacterial properties align with previous research, which has consistently demonstrated the potent antimicrobial potential of methanol as a solvent for phytochemical

extraction. This efficacy is attributed to methanol's ability to solubilize a broad spectrum of bioactive compounds, thereby enhancing the extract's antimicrobial action.^{17,28}

The study's outcomes corroborate the traditional use of *Rhus vulgaris* in treating infections and highlight its potential as a source of natural antibacterial agents. The extracts were more effective against gram-positive bacteria, a trend observed in other studies

examining the antibacterial properties of plant extracts.^{29,30} The methanol extract's effectiveness suggests it contains a higher concentration of active phytochemicals capable of penetrating the peptidoglycan layer of gram-positive bacteria.^{29,31}

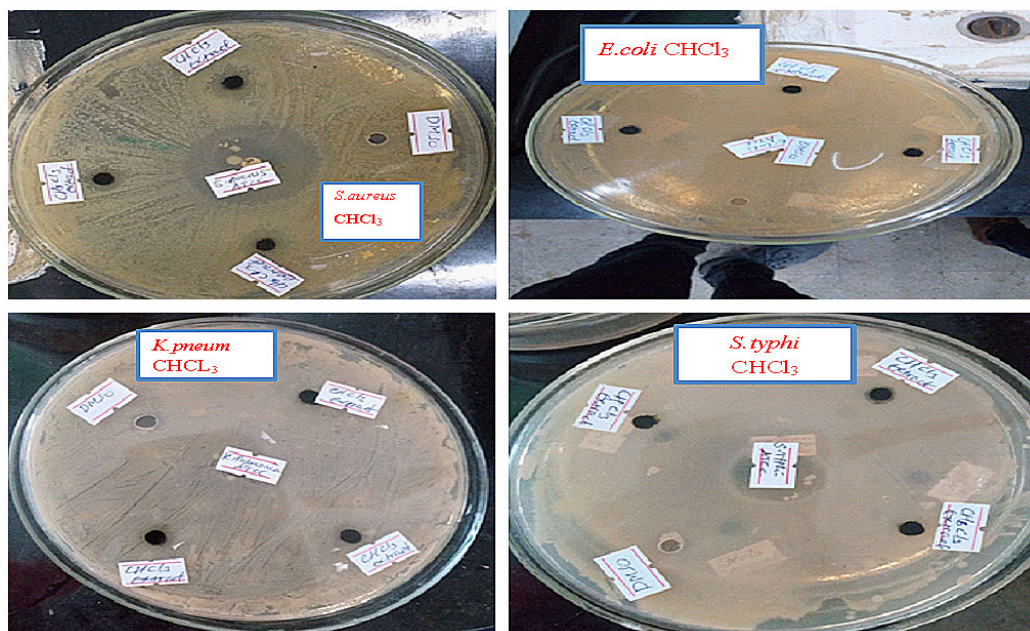


Fig. 4: Zone of inhibition of chloroform compared with an inhibition zone of standard antibiotic Gentamicin on *S. aureus*, *S. Typhi*, *K. pneumonia*, and *E. coli*.

Characterization of Compounds

In characterizing the compounds isolated from the leaves of *Rhus vulgaris*, we utilized both the RF value and spectroscopic data of the compounds.

Partial Characterization of AA₁

Compound AA₁, a dark green gummy substance, was isolated from the leaves of *Rhus vulgaris*. It exhibited a pink hue under a UV lamp and turned yellow upon exposure to 4% vanillin H₂SO₄. In methanol: chloroform (1:8), its R_f value was measured at 0.70.

FTIR Spectral Analysis

The FTIR spectrum of compound AA₁ (Figure 5) revealed an absorption band at 3454 cm⁻¹, indicating the presence of the -OH group. Additionally, bands observed at 3000 cm⁻¹, 2912 cm⁻¹, and 2882 cm⁻¹ corresponded to the stretching of aliphatic =C-H, CH₂, and CH₃ groups, respectively. Furthermore,

a band at 1637 cm⁻¹ indicated C=C bending. The bands observed at 1464 cm⁻¹, 1355 cm⁻¹, and 1078 cm⁻¹ were assigned to CH₂ bending, CH₃ bending, and C-O stretching vibrations, respectively.

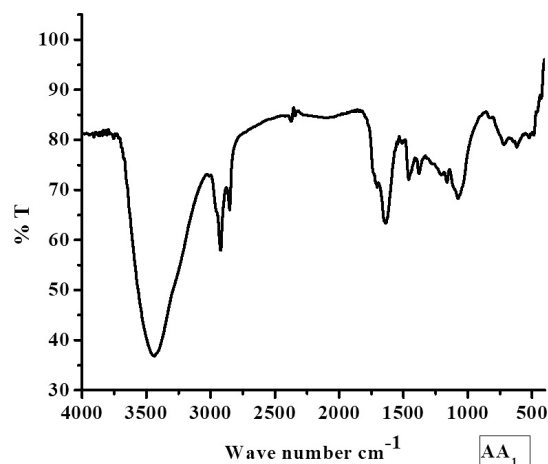


Fig. 5: FT-IR Spectrum of compound AA₁.

¹H-NMR Spectrum

In Figure 6, the peak at δ 1.25-2.07 (m) corresponds to the protons of the nine methylene groups, appearing as a multiplet due to signal overlap. Additionally, peaks at δ 0.84 (t) and δ 2.35 (d) indicate

the signals of the methyl group protons. The peak at δ 4.50-6.03 (t) indicates the protons of the five methine groups, while δ 6.99-7.07 (d) indicates the protons of the four methine groups.

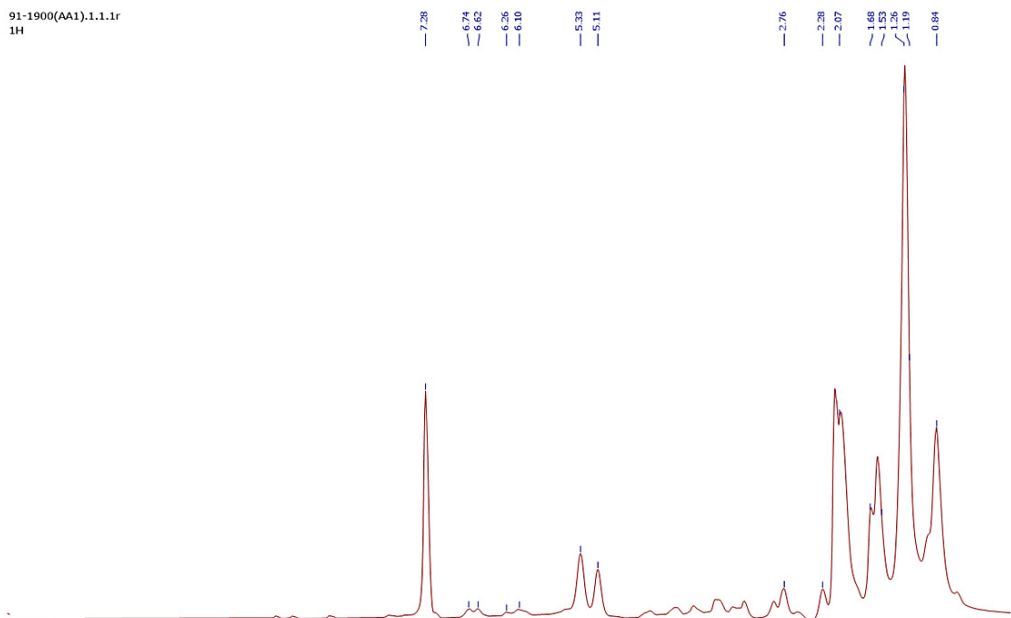


Fig. 6: ¹H-NMR spectrum of compound AA1.

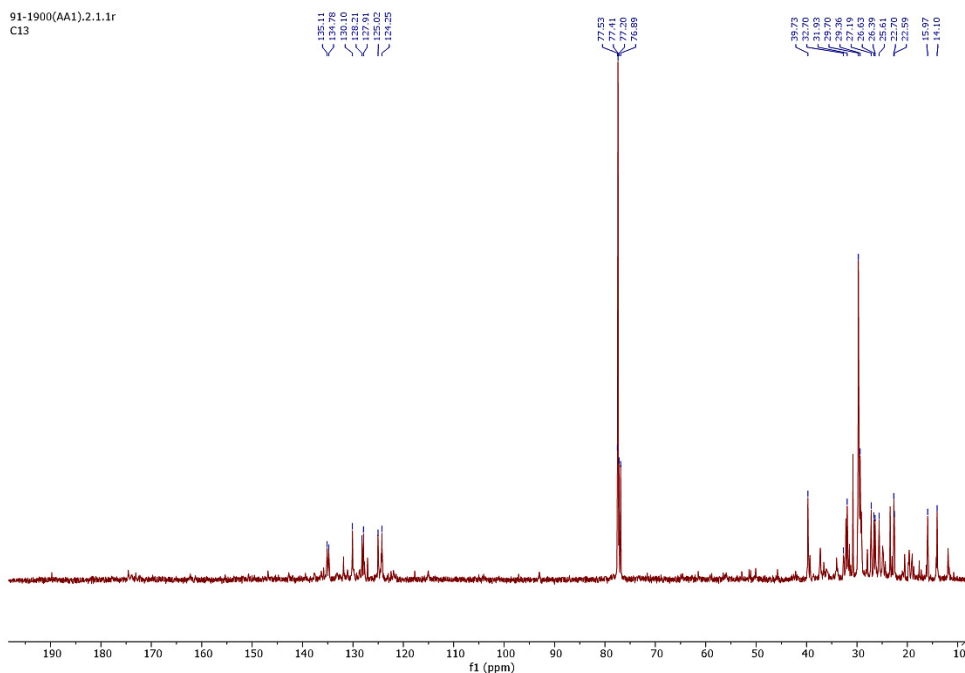


Fig. 7: ¹³C NMR spectrum of compound AA1.

¹³C-NMR Spectrum

The proton-decoupled ¹³C-NMR spectrum in Table 5 and Figure 7 displayed signals for 17 carbon atoms, whereas the proposed structure (AA1) contains 22 carbon atoms. The variance in the number of carbon atoms between the proposed structure and the signals detected in the ¹³C NMR spectrum might be attributed to the presence of chemically or magnetically equivalent carbon atoms. As depicted in Figure 9, carbon atoms 4, 5, 12, 6, 11, 8, 9, 10, 3', 1', 4', and 2' & 6' could be chemically equivalent.

The DEPT spectrum in Figure 8 exhibited signals for 11 carbon atoms, with 4 signals indicating the presence of 9 methylene groups and the remaining 7 signals for CH and CH₃ groups. In the DEPT spectrum, data are acquired in a manner that results in signals being either upfield (CH & CH₃) or downfield (CH₂), depending on the number of attached protons. In contrast, the proton-decoupled ¹³C NMR spectrum depicted in Figure 7 displayed signals for 22 carbon atoms, while the DEPT-135 spectrum revealed an overlap of signals for 20 carbon atoms.

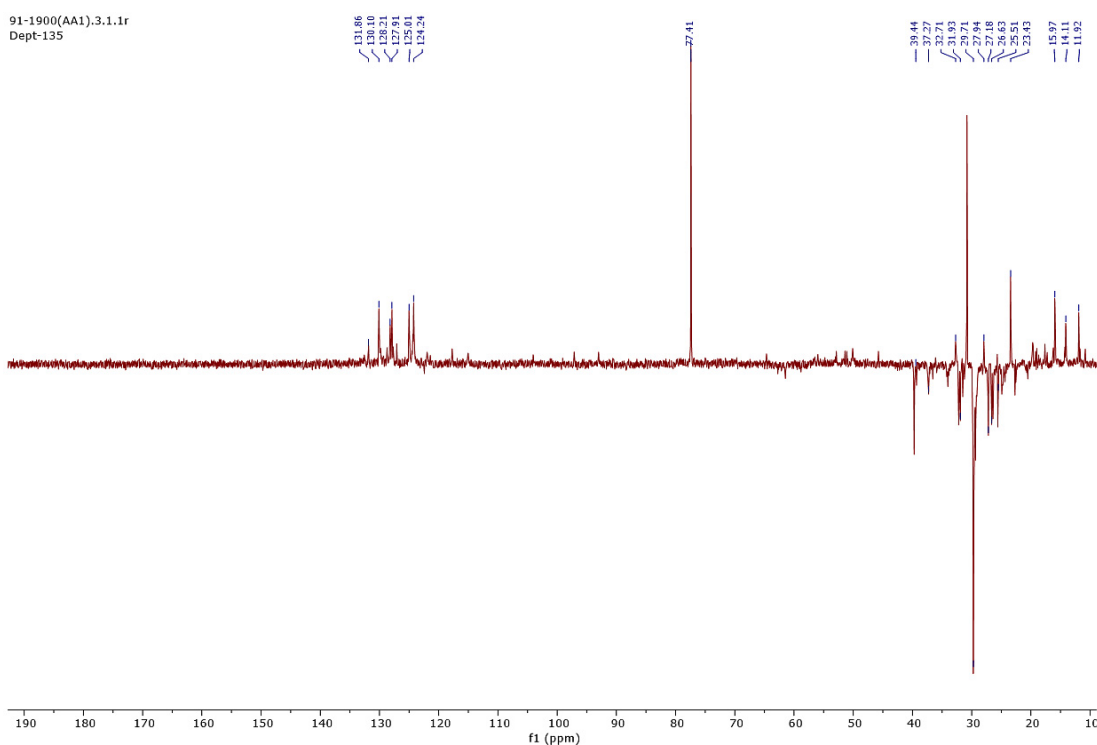


Fig. 8: DEPT-135 spectrum of compound AA1.

Table 5: ¹³C NMR and DEPT-135 spectra data of compound AA1

NO.	¹³ C NMR of AA1 δ (ppm)	DEPT	Remark
1	76.89	CH	-
2	39.73	CH ₂	-
3	22.70	CH ₂	-
4	29.70	CH ₂	-
5	29.70	CH ₂	-
6	32.70	CH ₂	-
7	135.70	CH	-

8	128.21	CH	-
9	128.21	CH	-
10	128.21	CH	-
11	32.70	CH ₂	-
12	29.70	CH ₂	-
13	32.70	CH ₂	-
14	22.59	CH ₂	-
15	14.10	CH ₃	-
1'	135.11	-	Quaternary
2'	127.91	CH	-
3'	128.21	CH	-
4'	135.11	-	Quaternary
5'	128.71	CH	-
6'	127.91	CH	-
7'	25.61	CH ₃	-

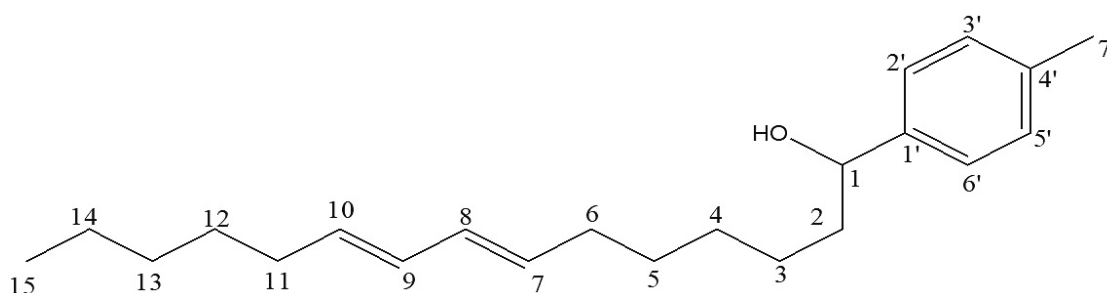


Fig. 9: The proposed structure of AA1 (1-p-tolyl pentadeca-7,9-dien-1-ol).

The disparity in signals between the two spectra suggested the presence of 2 quaternary carbon atoms that are typically not observed in the DEPT-135 spectrum. The ¹³C and DEPT chemical shifts of the proposed structure are summarized in Table 5 below.

Conclusion

In conclusion, n-hexane (4.35 g), chloroform (14.7 g), and methanol (100.7 g) extracts were obtained from *Rhus vulgaris*. Qualitative phytochemical screening of these crude extracts revealed the presence of alkaloids, glycosides, steroids, anthraquinones, and carbohydrates. Bioactivity assays demonstrated that methanol extracts, containing these bioactive compounds, exhibited significant antibacterial activity, with notable inhibition zones observed. Specifically, the methanol extract showed the highest potency as an antibacterial agent, effectively inhibiting the growth of both *Staphylococcus aureus* and *Salmonella typhi*. In contrast, chloroform and n-hexane extracts displayed moderate effectiveness

against *S. aureus* and *Escherichia coli*, but showed no antibacterial activity against *Klebsiella pneumoniae* and *S. typhi* compared to Gentamicin.

Furthermore, the methanol extract was subjected to column chromatography, leading to the isolation and structural elucidation of compound AA1 (1-p-tolyl pentadeca-7,9-dien-1-ol) using chromatographic methods, FTIR, and 1D NMR spectroscopic techniques. These findings highlight the potential of advanced chromatographic techniques such as HPLC and GS-MS for isolating additional compounds from various plant extracts. Additionally, the application of 2D-NMR and MS techniques is recommended for further elucidating the structures of novel compounds isolated from *Rhus vulgaris*. Future research should include comprehensive bioassay tests on crude extract fractions and isolated compounds to fully evaluate their antibacterial efficacy and potential therapeutic applications.

Acknowledgment

The authors express their sincere appreciation to Abreham Tesfaye Besha for the assistance provided in preparing this manuscript. The authors are grateful for the workshop facilities provided by the University of Gondar (Ethiopia) and Jigjiga University (Ethiopia). I would like to thank the Department of Chemistry at Addis Ababa University and the Faculty of Chemical and Food Science at Bahir Dar University for running the NMR and FTIR spectra analysis of my samples respectively and also the Institute of Biotechnology, the University of Gondar for their contribution in antibacterial analysis.

Funding Sources

Funding is not applicable to this study.

Conflict of Interest

The author(s) do not have any conflict of interest.

Ethics Statement

All methods are carried out according to the institution's guidelines and regulations.

Authors' contribution

AA - Study design, Literature search, data collection, data analysis, data interpretation, writing manuscript, GG - Research supervision, Study design, editing manuscript, DS – Research supervision, editing manuscript. All authors read and approved the final manuscript.

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