

Isolation and Characterization of an Acyclic Isoprenoid from *Semecarpus anacardium* Linn. and its Antibacterial Potential *in vitro*

- Antimicrobial Activity of *Semecarpus anacardium* Linn. Seeds -

Ayyakkannu Purushothaman^{1*}, Packirisamy Meenatchi¹, Nallappan Saravanan², Muthu Karuppaiah³, Ramalingam Sundaram^{1,4*}

¹ PG & Research Department of Biochemistry, Mohamed Sathak College of Arts and Science, Tamil Nadu, India

² Department of Zoology, Presidency College (Autonomous), Tamil Nadu, India

³ Department of Chemistry, Manomanium Sundaranar University, Tirunelveli, Tamil Nadu, India

⁴ Scientist Grade- III, Central Research Laboratory, Meenakshi Ammal Dental College and Hospital, Maduravoyal, Tamil Nadu, India

Key Words

acyclic isoprenoid, antibacterial, disk diffusion method, high-resolution mass spectrometry, *Semecarpus anacardium* Linn.

Abstract

Objectives: *Semecarpus anacardium* Linn. is a plant well-known for its antimicrobial, antidiabetic and anti-arthritis properties in the Ayurvedic and Siddha system of medicine. This has prompted the screening of this plant for antibacterial activity. The main aims of this study were to isolate compounds from the plant's seeds and to evaluate their antibacterial effects on clinical bacterial test strains.

Methods: The n-butanolic concentrate of the seed extract was subjected to thin layer chromatography (TLC) and repeated silica gel column chromatography followed by elution with various solvents. The compound was identified based on observed spectral (IR, ¹H NMR, ¹³C NMR and high-resolution mass spectrometry) data. The well diffusion method was employed to evaluate the antibacterial activities of the isolated acyclic isoprenoid compound (final concentration: 5 - 15 µg/mL) on four

test bacterial strains, namely, *Staphylococcus aureus* (MTCC 96), *Bacillus cereus* (MTCC 430), *Escherichia coli* (MTCC 1689) and *Acinetobacter baumannii* (MTCC 9829).

Results: Extensive spectroscopic studies showed the structure of the isolated compound to be an acyclic isoprenoid (C₂₁H₃₂O). Moreover, the isoprenoid showed a remarkable inhibition of bacterial growth at a concentration of 15 µg/mL compared to the two other doses tested (5 and 10 µg/mL) and to tetracycline, a commercially available antibiotic that was used as a reference drug.

Conclusion: The isolation of an antimicrobial compound from *Semecarpus anacardium* seeds validates the use of this plant in the treatment of infections. The isolated compound found to be active in this study could be useful for the development of new antimicrobial drugs.

1. Introduction

Plants have formed the basis of the traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Ayurveda is, perhaps, the most ancient of all

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*Corresponding Author

Dr. A. Purushothaman Head of the Department, PG & Research Department of Biochemistry, Mohamed Sathak College of Arts and Science (Affiliated to the University of Madras), Chennai-600 119, Tamil Nadu, India. Dr. R.Sundaram, Scientist Grade- III, Central Research Laboratory, Meenakshi Ammal Dental College and Hospital, Maduravoyal, Chennai-600 095, Tamil Nadu, India.
E-mail: purushothamanbiochem@gmail.com, rsundaram77@yahoo.co.in

medicinal traditions. It is actually a practical and holistic set of guidelines to maintain balance and harmony in the body [1]. *Semecarpus anacardium* Linn. (Family: Anacardiaceae) is a plant well-known for its medicinal value in the Ayurvedic and Siddha system of medicine. It is distributed in the sub-Himalayan region, especially in the tropical and central parts of India. The nut is commonly known as the 'marking nut' and in the vernacular as 'Ballataka' or 'Bhilwa' [2, 3]. Chemical and phytochemical analyses of this nut have revealed the presence of bioflavonoids, phenolic compounds, bhitlanols, minerals, vitamins and amino acids. A variety of nut extract preparations from this plant have been reported to have anti-atherogenic, anti-inflammatory, antioxidant, anti-reproductive, central nervous system (CNS) stimulating, hypoglycemic, and anti-carcinogenic activities [4].

Due to the indiscriminate application of antibacterial drugs, many microbial organisms have developed high resistance to a number of antibiotics. This, coupled with other problems like the dangerous side effects of some commercial antibiotic drugs, has led scientists to think of other alternatives like new antimicrobial substitutions from other sources, especially medicinal plants [5]. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care [6]. According to the World Health Organization (WHO), medicinal plants should be the best sources from which to obtain a variety of drugs. Therefore, such plants should be investigated to obtain a thorough knowledge about their properties, safety and efficacy [7].

Semecarpus anacardium Linn, belongs to the family Anacardiaceae, and in indigenous systems of medicine, its seeds have high priority and applicability for treating various ailments. However, the mechanism of the pharmacological action of its nuts can be established only by the isolation of its active components and determinations their structures and functions. This background provided the motivation for our attempting to isolate the active components of *Semecarpus anacardium* seeds and to determine the effects of those active components on both human pathogenic gram positive bacteria, such as *Bacillus cereus* MTCC 430 and *Staphylococcus aureus* MTCC96, and on human pathogenic gram negative bacteria, such as *Escherichia coli* (*E. coli*) MTCC 1689 and *Acinetobacter baumannii* MTCC 9829, by using the well diffusion method [8].

2. Materials and Methods

The thin layer chromatography (TLC) aluminum sheet and 20 × 20 silica gel 60 F254 were obtained from Merck, Darmstadt Germany. Silica gel, 60 - 120 mesh size, for column chromatography, chloroform, methanol, diethyl ether, n-butanol, hexane and ethyl acetate were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All chemicals used were of analytical grade. *Semecarpus anacardium* seeds were purchased from Ramasamy Chettiyar, Traditional & Herbal Medicine shop, Parrys, Chen-

nai-600 001, Tamil Nadu, India. The identity of the plant was confirmed by Prof. Raman, Plant Taxonomist, Centre for Advanced Studies in Botany, University of Madras, and voucher specimens (MUCASB-H105) was deposited in the department's herbarium.

Five hundred gram of *Semecarpus anacardium* seeds were crushed, soaked in a liter of methanol, and then kept in a refrigerator for 3 days. Then, the filtrate was filtered through Whatman filter paper No. 1; this was repeated three to four times until the filtrate showed no coloration, after which it was concentrated using a vacuum rotary evaporator at 40°C. The methanolic concentrate was fractionated sequentially with petroleum, diethyl ether, chloroform and n-butanol. The n-butanolic fraction was evaporated to dryness. Before the active compound was isolated from the seeds of *Semecarpus anacardium*, the n-butanolic fraction of the *Semecarpus anacardium* seeds was subjected to preliminary phytochemical screening using the standard procedures of Harborne [9] and Kokate [10] to determine its chemical constituents.

After the chemical constituents in the extract of *Semecarpus anacardium* seeds had been identified, the extract was analyzed using TLC. The n-butanolic concentrate was subjected to TLC using hexane and ethyl acetate in a ratio of 8 : 2 as a mobile phase, and four spots appeared. The n-butanolic concentrate was chromatographed on a silica gel column (Merck 60 -120 mesh, 750 g, 3.5 i.d. x 60 cm) and eluted successively with hexane and ethyl acetate (80 : 20 ratio). A total of 50 fractions were collected at intervals of 10 mL and monitored by using TLC (precoated silica gel merk-60 F254 0.25-mm-thick plate). Fractions from 1 to 5, for which pale green or straw yellow color was observed and which showed a single spot on TLC, were pooled together in a clean vial and evaporated to dryness. This process was repeated until a satisfactory yield of each compound had been achieved. The structure of each compound was confirmed on the basis of infrared (IR), ¹H nuclear magnetic resonance (NMR), ¹³C NMR, and high-resolution mass spectrometry (HRMS).

Both human pathogenic positive bacteria, i.e., *Bacillus cereus* (MTCC 430) and *Staphylococcus aureus* (MTCC 96), and human pathogenic negative bacteria, i.e., *E. coli* (MTCC 1687) and *Acinetobacter baumannii* (MTCC 9829), were chosen based on their clinical and pharmacological importance. The bacterial strains were obtained from the Institute of Microbial Technology, Chandigarh, India, and were used for evaluating antimicrobial activity. All the bacterial strains were maintained on nutrient agar in slants or Petri Plates at room temperature (28 ± 2°C). Isolated compound in the concentration range from 5 to 15 µg/mL dissolved in 10% dimethyl sulfoxide (DMSO) were used in this study, and tetracycline was used as a reference drug. The value of the minimum inhibitory concentration (MIC) was taken as the lowest concentration of the compound that showed prominent inhibition of bacterial growth after a 24-h incubation at 37°C.

UV spectra were recorded with a UV160A- Shimadzu spectrophotometer. The IR spectra were recorded with a Thermo Satellite fourier transform infrared spectroscopy (FT-IR) spectrophotometer. The ¹H and the ¹³C NMR spectra were recorded using a 300- and 75.1-MHz Bruker

Table 1 Phytochemical screening of the *Semecarpus anacardium* seed extract

No	Test	Observation	Inference
1	Alkaloids	Brown precipitate formed	+
2	Flavonoids	Reddish pink color was observed	+
3	Saponins	Layer of foam did not form White precipitate did not form	–
4	Carbohydrates	A brown ring was observed	+
5	Phenols	Blue green color was observed	+
6	Triterpenoids	Reddish violet color was observed A purple color was not observed	–
7	Phytosterols	Bluish green color was observed Red color was observed	+
8	Glycosides	Red color was observed Pink color was observed	+

+, indicates presence; –, indicates absence.

spectrometer, respectively, with CDCl_3 (deuterated chloroform) as the solvent, and chemical shifts were recorded in parts per million with tetramethylsilane (TMS) as an internal reference. The mass spectra were obtained using QTOF mass spectrometers. Column chromatography (CC) was performed on silica gel 60 - 120 mesh (Merck). TLC plates precoated with silica gel 60 and fluorescent indicator F254 were used for analytical purposes. The data were analyzed by using simple arithmetic means and were expressed as means \pm standard deviations (SDs).

3. Results

Phytochemical screening of the extract revealed the presence of alkaloids, flavonoids, carbohydrates, phenols, steroids, and glycosides (Table 1). The n-butanolic concentrate was subjected to TLC using hexane and ethyl acetate in the ratio of 80 : 20 as a mobile phase, and four spots appeared (Fig. 1). The structure of the compound was confirmed as an acyclic isoprenoid derivative on the basis of IR (Fig. 2), ^1H NMR (Fig. 3), ^{13}C NMR (Fig. 4), and HRMS (Fig. 5) measurements. The molecular formula of the compound is $\text{C}_{21}\text{H}_{32}\text{O}$. The yield of the compound was 300 mg/500 g of crude methanolic extract. The chemical structure of the acyclic isoprenoid is given in Fig. 6.

The FTIR spectrum of the acyclic isoprenoid showed an absorption peak at $1,754\text{ cm}^{-1}$, which could be assigned



Figure 1 Isolation of acyclic isoprenoid from the butanolic fraction of *Semecarpus anacardium* seeds by using TLC and column chromatography. (Mobile Phase: hexane : ethyl acetate in the ratio of 8 : 2).

A, TLC plate showing four spots; B, separation using column chromatography; C, TLC plate showing isoprenoid; D, Vial containing acyclic isoprenoid.

TLC, thin layer chromatography.

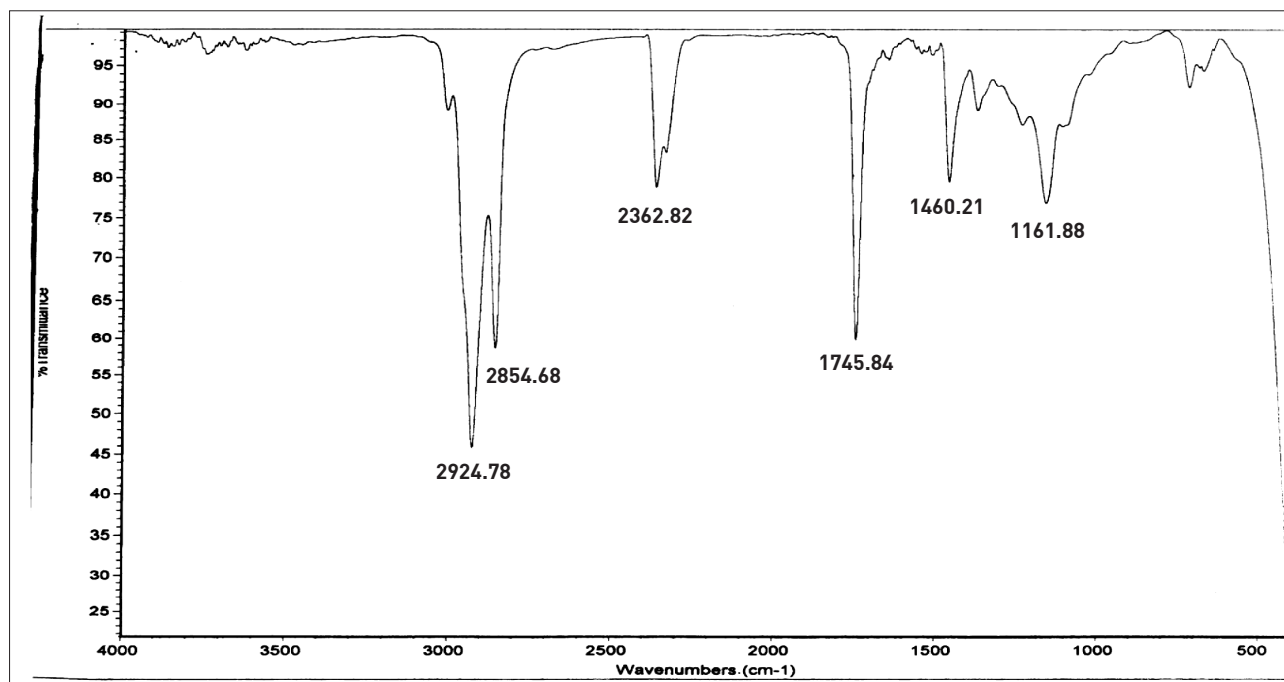


Figure 2 FTIR spectrum data for the acyclic isoprenoid.

FT-IR, Fourier transform infrared spectroscopy.

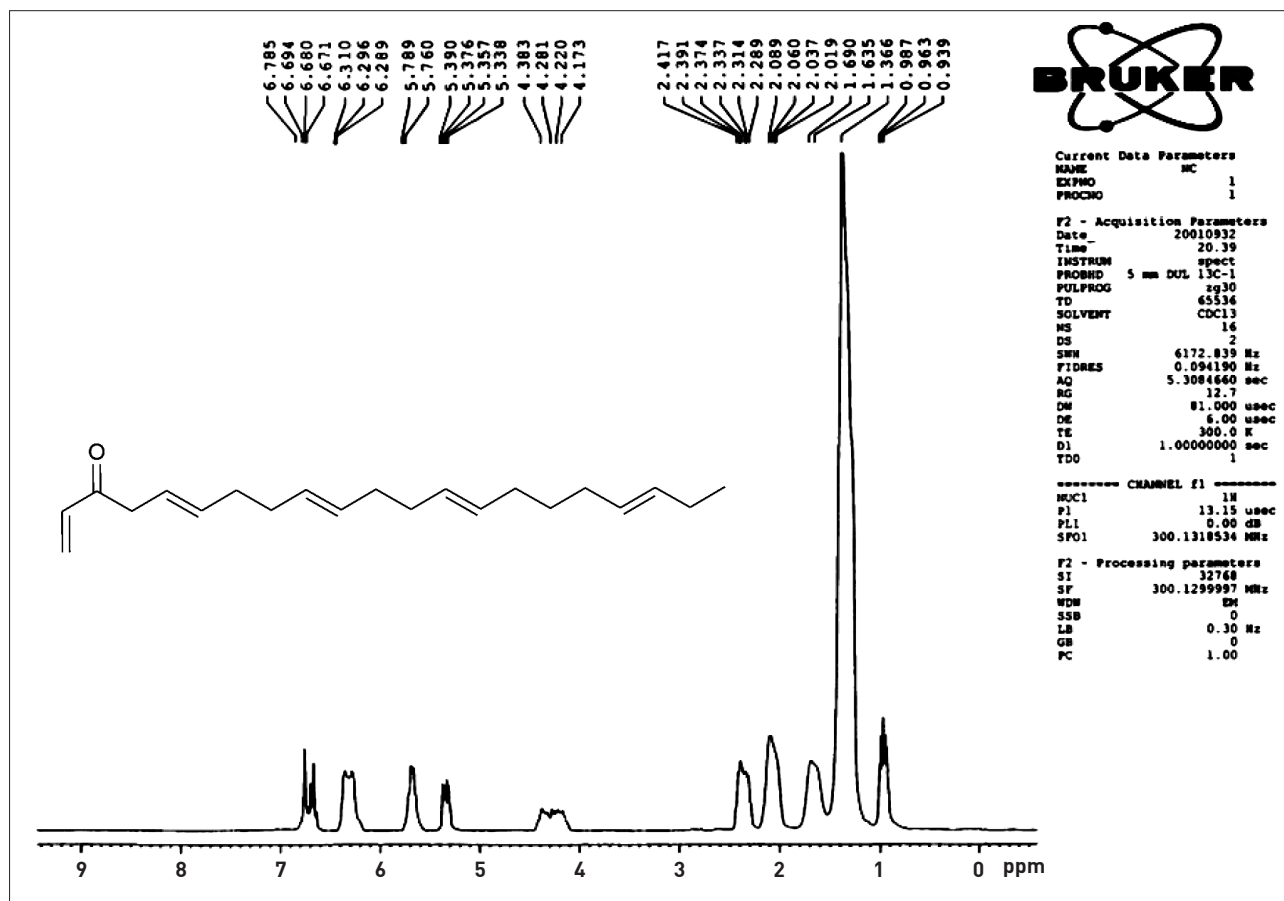


Figure 3 ¹H NMR spectrum data for the isolated acyclic isoprenoid.

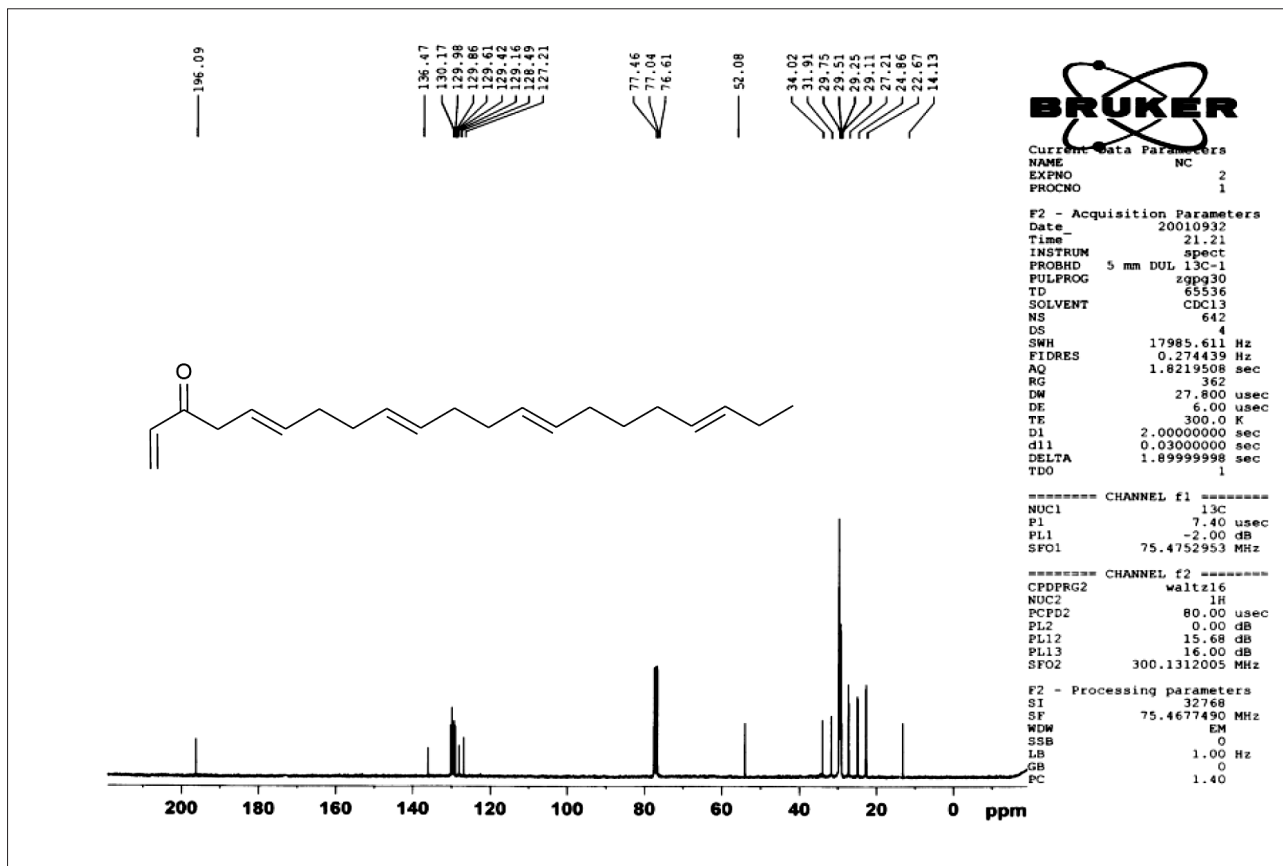
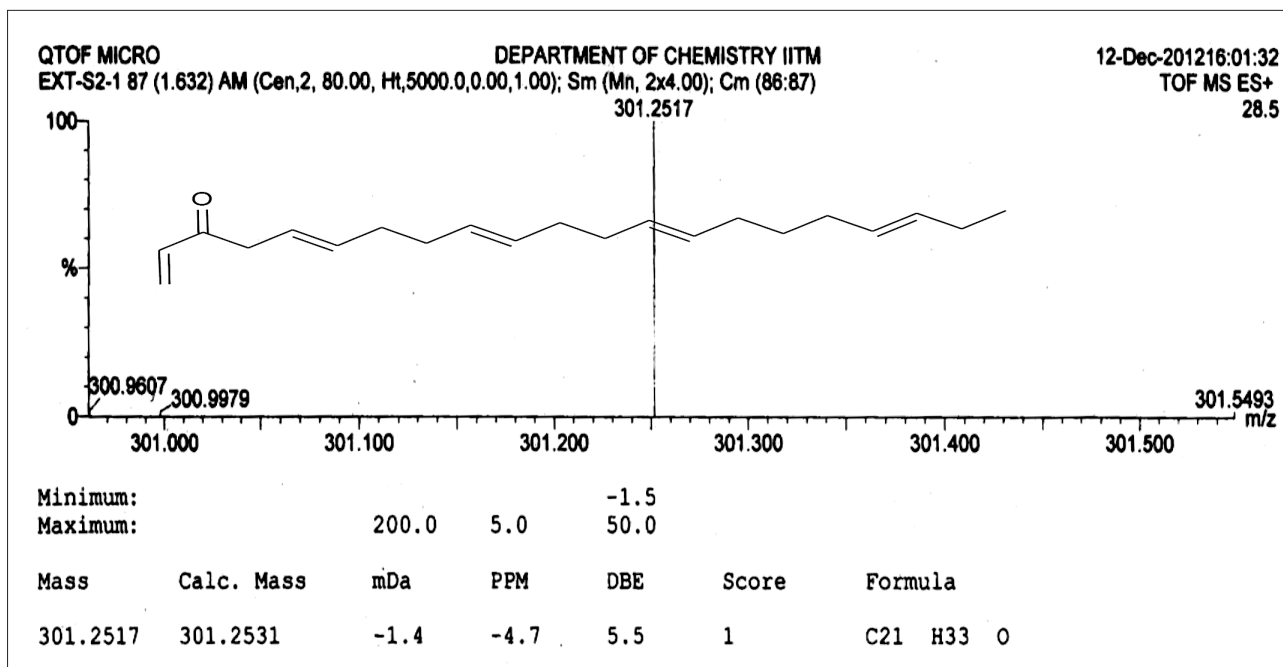
Figure 4 ¹³C NMR spectrum data for the isolated acyclic isoprenoid.

Figure 5 HR-MS data for the isolated acyclic isoprenoid.

HR-MS, High-resolution mass spectrum.

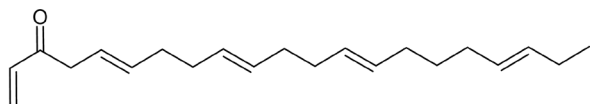


Figure 6 Chemical structure of the acyclic isoprenoid.

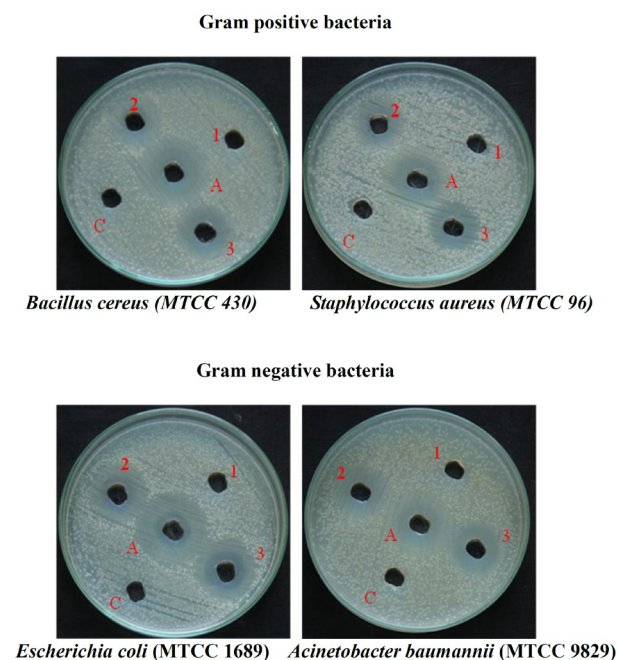


Figure 7 The antibacterial activity of the studied isolated isoprenoid against both gram positive (*Staphylococcus aureus* MTCC96 and *Bacillus cereus* MTCC 430) and gram negative (*E. coli* MTCC 1689 and *Acinetobacter baumannii* MTCC 9829) bacteria.

A, positive control, tetracycline (15 µg/mL); C, negative control (10% DMSO); 1, isoprenoid (5 µg/mL); 2, isoprenoid (10 µg/mL); 3, isoprenoid (15 µg/mL).

DMSO, dimethyl sulfoxide.

as being due to a carbonyl group. The other peaks at 2,924 and 2,854 cm^{-1} were assigned to methyl and methylene stretching groups, respectively. The ^1H NMR spectrum showed a triplet signal at $\delta = 0.96$ (3H, t, $J = 7.2$ Hz), which corresponded to the methyl protons present in the isoprenoid compound. The alkene protons appeared as a multiplet at $\delta = 6.28 - 6.31$, which was adjacent to the peak associated with the carbonyl group. The ^{13}C NMR spectrum of the isoprenoid showed the presence of 21 carbon signals. The peaks at $\delta = 196.09$ and 14.13 were identified as being due to the carbonyl carbon and the methyl carbon groups, respectively. The isoprenoid compound was assigned the molecular formula $\text{C}_{21}\text{H}_{32}\text{O}$ based on the HRMS (EI) molecular ion peak at $m/z = 301.2517$ [M+].

Figs. 2 - 5 show the spectral values of the isoprenoid's IR (KBr) λ_{max} : 2924 (-CH₃), 2854 (= CH), 1745 (C = O), 1,460, and 1,161 cm^{-1} . From ^1H NMR (300 MHz, CDCl_3), $\delta = 0.96$ (t, 3H $J = 7.2$ Hz), 1.36 (s, 2H), 1.63 - 1.69 (m, 2H), 2.01 - 2.09 (m, 2H), 2.29 - 2.42 (m, 8H), 4.17 - 4.38 (m, 2H), 5.33 - 5.39 (m, 6H), 5.76 - 5.79 (m, 4H), 6.29 - 6.31 (m, 1H), and 6.67 - 6.79 (m, 2H). From ^{13}C NMR (75 MHz, CDCl_3), $\delta = 14.13$ (C-21), 22.67 (C-16), 24.86 (C-20), 27.21 (C-17), 29.11 (C-15), 29.25 (C-11), 29.51 (C-12), 31.91 (C-7), 34.02 (C-8), 52.08 (C-4), 127.21 (C-1), 128.49 (C-14, C-18), 129.16 (C-6), 129.42 (C-13), 129.61 (C-9), 129.86 (C-10), 129.98 (C-19), 130.17 (C-5), 136.47 (C-2), and 196.09 (C-3).

The antibacterial activities of the studied isoprenoid against both gram positive (*Staphylococcus aureus* MTCC96 and *Bacillus cereus* MTCC 430) and gram negative (*E. coli* MTCC 1689 and *Acinetobacter baumannii* MTCC 9829) organisms at different concentrations ranging from 5 to 15 µg/mL and their bacterial activities were compared to those of the reference control (tetracycline). The antibacterial activity of isoprenoid was found to increase with increasing concentration against all bacterial strains tested, as evidenced by the higher zones of inhibition at higher concentrations (Fig. 7). Moreover, isoprenoid showed a remarkable inhibition of bacterial growth at a concentration of 15 µg/mL compared to the other two doses (5 and 10 µg/mL) and to tetracycline, a commercially available antibiotic drug that was used as the reference control drug (Table 2).

Table 2 Antimicrobial activity of isoprenoid against pathogenic microorganisms

	Gram positive		Gram negative	
	<i>B. cereus</i> MTCC 430	<i>S. aureus</i> MTCC 96	<i>E. coli</i> MTCC 1689	<i>A. baumannii</i> MTCC 9829
A: positive control (15 µg/mL)	26 ± 1.03	19 ± 1.21	31 ± 1.42	18 ± 1.11
C: negative control (10% DMSO)	NA	NA	NA	NA
1: acyclic isoprenoid (5 µg/mL)	NA	NA	NA	NA
2: acyclic isoprenoid (10 µg/mL)	14 ± 0.98	12 ± 1.08	16 ± 0.73	11 ± 0.49
3: acyclic isoprenoid (15 µg/mL)	19 ± 1.13	16 ± 0.93	22 ± 1.21	13 ± 0.85

Values are means ± SDs of three replicates.

DMSO, dimethyl sulfoxide; NA, no activity exhibited against microorganism; SDs, standard deviations.

4. Discussion

Infectious bacterial diseases represent an important cause of morbidity and mortality worldwide. Therefore, the development of new antimicrobial agents for the treatment of bacterial infections is of increasing interest. A number of naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions, and they may serve as sources of antimicrobial agents against pathogens [11]. In this study, a compound was isolated from *Semecarpus anacardium* seeds by using column and thin layer chromatography and was characterized as isoprenoid by using IR, HR mass, ^1H , and ^{13}C NMR spectroscopy methods.

Plants are important sources of potentially functional structures for the development of new therapeutic agents. The first step towards this goal is an *in-vitro* antibacterial activity assay [12-14]. In the present study, a compound was isolated, and the structure was elucidated as acyclic isoprenoid ($\text{C}_{21}\text{H}_{32}\text{O}$) by using extensive spectroscopic studies. Earlier studies have documented the antimicrobial activity of isoprenoids, such as geraniol, geranic acid, geranyl acetate, geranyl formate, farnesol, farnesal, citral, citronellol, linalool, linalyl acetate, citronellal, 2,6-diepoxygeraniol, nerol, and terpinen-4-ol, towards gram positive and gram negative bacterial and fungal species [15]. Jeong *et al* reported that the catechol derivative from *Diospyros kaki* showed antibacterial activity against *E. coli* and *Lactobacillus casei* [16]. Moreover, the alcoholic extracts of dry nuts, leaves, and green fruits of *Semecarpus anacardium* are reported to have antibacterial activity against both gram negative and gram positive human pathogenic organisms [17].

The isoprenoid compound was significantly active, exhibiting antimicrobial activity against tested organisms viz. *Bacillus cereus* (MTCC 430), *Staphylococcus aureus* (MTCC 96), *E. coli* (MTCC 1687) and *Acinetobacter baumannii* (MTCC 9829). The antibacterial activity of isoprenoid was found to increase with increasing concentration against all bacterial strains tested, as evidenced by the higher zones of inhibition at higher concentrations. Moreover, isoprenoid showed a remarkable inhibition of bacterial growth at a concentration of 15 $\mu\text{g}/\text{mL}$ compared to the other two doses (5 and 10 $\mu\text{g}/\text{mL}$) and to tetracycline, a commercially available antibiotic drug that was used as a reference control drug. This is interesting in view of the perspective of developing new antibacterial drugs from natural products. To the best of our knowledge, this is the first report on the antimicrobial activities of the isoprenoid compound isolated from *Semecarpus anacardium* seeds.

The overall results of this study can be considered as very promising in the perspective of obtaining new drugs from plant sources, especially when the medical importance of the tested microorganisms is considered. *Staphylococcus aureus* is a major cause of community and hospital-associated infections, with an estimated mortality of around 7% - 10% [18]. Moreover, about 2% of patients in Cameroon are infected with *Staphylococcus* spp. [19]. Each year, some 500,000 patients in American hospitals contract a staphylococcal infection [18]. Such findings stress the importance of finding an antibiotic against which the *Staphylococcus*

aureus organism is sensitive. This pathogen was found to be sensitive to the isolated compound.

5. Conclusion

The antimicrobial compound isolated from *Semecarpus anacardium* seeds validates the use of this plant in the treatment of infections. Furthermore, the isolated compound found to be active in this study could be useful for the development of new antimicrobial drugs. However, pharmacological and toxicological studies and research to find its mechanisms of action, which are currently going on in our laboratory, will be necessary to confirm this hypothesis.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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