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Research Article

Anti-Staphylococcus Aureus Guide Isolation of Compounds from the Fruits of *Ziziphus Mauritiana*

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Abstract

The present work deals with the isolation, purification and characterization of secondary metabolites of the fruits of Ziziphus mauritiana and the evaluation of their biological activity against Staphylococcus aureus. A plant from the Rhamnaceae family, widely used in the Cameroonian and Tchadian traditional pharmacopoeia for the treatment of various diseases such as food poisoning, pneumonia, urinary tract infections, and skin infections such as wounds and ulcers. Our investigations focused on the methanol/dichloromethane (MeOH/DCM, 1:1) extract of the fruits of Ziziphus mauritiana. Alkaloidal treatment was performed on this extract, and we obtained four (04) fractions: two non-alkaloid fractions, F1 (DCM), F2 (EtOAc), and two alkaloid fractions, F3 (DCM) and F4 (EtOAc). Using usual chromatographic methods (CC, CCM), we isolated from the alkaloidal fraction F3, two compounds 1 and 2, and four compounds 3, 4, 5, 6 from the non-alkaloid fraction F1, the hexane fraction. From these compounds, three (03) were fully characterized using spectroscopic methods 1H and 13C NMR (1 and 2 dimensions) and by comparison of their spectral data with those of the literature. These are: Sanjoinenine 1, betulinic acid 3, and epicatechin 4. The crude extract as well as fraction, and some isolated compounds were evaluated in vitro by the liquid microdilution method described by CLSI, against two strains of Staphylococcus aureus: S. aureus ATCC25923, S. aureus ATCC43300, and a Clinical isolate. The crude extract, fractions F1 and F4, were the most active fractions tested against the strain S. aureus ATCC 25923, with a MIC of 15.6, 62.5, and 15.6 µg/mL, respectively, while the other fractions (F2 and F3) showed moderate activity against the same strain. Fraction F2 is not active. For the S. aureus ATCC 43300, all the fractions have a weak activity, and for the clinical isolate, only ZSA shows a good activity with a MIC of 62.5 µg/mL, and the others show a moderate activity.

Keywords

Ziziphus Mauritiana, Staphylococcus Aureus, Sanjoinenine 1, Betulinic Acid 3, Epicatechin 4

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1. Introduction

Staphylococcus aureus is the most dangerous of the many *Staphylococci* commonly found. Among the diverse multi-drug-resistant bacteria, methicillin-resistant *Staphylococcus aureus* is probably the best-known resistant bacterium that has riveted intense scientific and political interest globally due to the limited spectrum of antibiotics for its effective treatment [1, 2]. Recent estimates of the World Health Organization revealed that the combined prevalence of MRSA in a diverse population of the African region is between 12 and 80% [3]. In Chad, Cameroon, and many other countries in Africa, the prevalence of MRSA has been slowly rising over the past few decades. In most of the hospitals, antibiotic treatment is not streamlined as per the microbiological culture data. Consequently, MRSA strains are becoming resistant not only to β-lactams but also to multiple antimicrobial agents, such as macrolides, aminoglycosides, fluoroquinolones, and [4]. From this standpoint, it is desirable to develop new antibiotic agents with reduced side effects and affordable to all.

Since immemorial time, the plant kingdom has catered to humans' need by providing a variety of drugs. Among them, terrestrial flora constitutes a prolific source of bioactive constituents. Plants synthesize a wide variety of metabolites to safeguard themselves and to maintain homeostasis in their environment. Investigations on naturally occurring antibiotic compounds from terrestrial plants have been carried out for more than a century [5]. Hitherto, more than 200,000 natural products have been recorded from diverse species of flora [6]. It has been purported that secondary metabolites from medicinal plants have minimal adverse reactions and can also repress the growth of pathogens by diverse mechanisms of action than the currently used synthetic antibiotics. Thus, medicinal plants such as *Ziziphus*, taken traditionally as decoction, tea, or infusion against bacterial diseases, may be welcome as a way to be explored.

The genus *Ziziphus* is known for its medicinal properties as a hypoglycemic, hypertensive, antiinflammatory, antimicrobial, antioxidant, antitumor, and liver protective agent and as an immune system stimulant [7]. According to Dr. Nidal A. Jaradat, in 2005, *Ziziphus* is used as food, a diuretic, for teeth decay, to increase hair growth, for antidiarrhea, and to decrease thirst [8].

Chemical studies carried out show that the genus Ziziphus, particularly the species mauritiana, fruits are nutritious, contain protein, carbohydrates, and vitamins. Carbohydrates constitute 80.6% in dry matter, particularly in starch (21.8%), sucrose (21.8%), glucose (9.6%), fructose (16%), and iron [9-14]. It has been reported that we can also find some secondary metabolites such as alkaloids, flavonoids, triterpenes, and cyclopeptides, which are predominant and have been shown to occur in different amounts in most Ziziphus species [10-14]. Several authors have worked on the subject, presenting different therapeutic forms or other aspects of the plant and its fruit [11, 12, 15-21].

2. Materiel et Method

2.1. General Experimental Procedures

Melting points were measured on a standard heating block melting point apparatus.

IR spectra were recorded on a Bruker Fourier transform/infrared (ATR) spectrophotometer. HR-ESI-MS spectra were measured with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. 1D (^{1}H, ^{13}C) and 2D (^{1}H-^{1}H-COSY, HSQC, HMBC) NMR spectra were recorded in deuterated solvents on a Bruker ARX 600 (^{1}H 600 MHz and ^{13}C 150 MHz). NMR spectrometer equipped with a 5 mm cryoprobe. All chemical shifts were measured in parts per million (ppm) using a residual solvent signal as secondary reference relatively to tetramethylsilane (TMS) as internal standard, while coupling constants (J) are given in Hz. Solvents were distilled prior to use. Analytical grade solvents were used for LC-MS. Column chromatography (CC) was performed using Merck MN silica gel 60 M (0.04-0.063 nm) and thin layer chromatography (TLC) was performed on aluminum silica gel 60 F_{254} (Merck) precoated plates (0.2 mm layer thickness). Spots were visualized on TLC either by UV lamp (254 and 365 nm) or by heating after spraying with 20% H_2SO_4 (v/v) solution. Different mixtures of *n*-hexane, EtOAc, DCM and MeOH were used as eluting solvents.

2.2. Plant Material

The seeds of Ziziphus mauritiana were collected in June 2021 at Ndjamena, a locality of Tchad, and were identified by Dr. ESSONO, a botanist. A specimen has been deposited at the National Herbarium.

2.3. Preparation of Stocks Solution of Extracts, Compounds and Reference Antibacterial

The antibacterial activity of extracts, fractions, and isolated compounds was performed using micro-dilution according to protocol M09-A7, The antibacterial activity of extracts, fractions and isolated compounds was performed using micro-dilution according to protocol M09-A7, coupled with a resazurin-based assay. Indeed, a serial dilution of extracts/fractions, compounds, and ciprofloxacin was performed in Muller Hinton Broth (Sigma Aldrich) to have different concentration ranges from 1000 to 1.95 µg/mL, from 125 to 0.48 µg/mL, and from 0.25 to 0.0078 µg/mL, respectively. The final bacteria load was 5 × 10^5 CFU/mL with the final volume of 200 µL. Ciprofloxacin was used as a positive control. Negative control constituted the culture medium and bacteria suspension, while sterility control was constituted with only culture media. All the plates were covered and incubated for 24 hrs at 37 ℃. At the end of the incubation period, 20 µL of freshly prepared resazurin (0.15 mg/mL) was added into each well and re-incubated under the same conditions for 30min. The

MIC was defined as the smallest concentration of extracts, fractions, or isolated compounds from which there is no change in coloration from blue to pink, corresponding to the absence of visible bacterial growth.

3. Results and Discussion

This study aimed to perform bio-guided isolation of the dichloromethane/methanol extract of the fruits of *Ziziphus mauritiana*. Extract and fractions were tested against two references strains of *S. aureus* namely *aureus* ATCC25923, *S. aureus* ATCC43300 and a clinical isolates *S. aureus CPC*. The following cut offs were used to classify the activity of samples: very active when MIC < 100 µg/ml; significantly active when 100 ≤MIC≤512 µg/ml; moderately active when 512 <MIC ≤2048 µg/ml; weakly active if MIC >2048 µg/ml and not active when MIC >10 000 µg/ml. The fruits crude extract exhibited significant activity with MICs values at 15.6, 62.5 and 15.6 µg/ml respectively. The crude extract was fractionated using Liquid partition. Four fractions were obtained and labeled from F1 to F4. (Table 1).

Table 1. *MIC values of anti-Staphylococcus aureus tests obtained on extract and fractions.*

	***S. aureus* ATCC25923**	***S. aureus* ATCC43300**	***S. aureus* Clinical isolate**
ZSA	15.6	125	62.5
F1	-	-	-
F2	250	500	250
F3	15.6	500	125
F4	125	500	250

SA ATCC 25923: *Staphylococcus aureus* AT 25923, SA ATCC 43300: *Staphylococcus aureus* ATCC 43300. ZSA: Crude extract, F1: non alkaloid fraction (DCM), F2: non alkaloid fraction (EtOAc), F3: alkaloid fraction (DCM), F4: alkaloid fraction (EtOAc).

The results obtained show that the crude extract (ZSA) and fraction F3 were the most active fractions tested against the strain *S. aureus* ATCC 25923, with a MIC of 15.6, 62.5, and 15.6 µg/ml, respectively, while the other fractions (F2 and F4) show moderate activity against the same strain; fraction F1 is not active. For the *S. aureus* ATCC 43300, all the fractions have a weak activity, and for the clinical isolate, only ZSA shows a good activity with a MIC of 62.5 µg/mL, and the others show a moderate activity. This can be explained by the synergetic effect of compounds inside the crude extract. These results could also be justified by the richness of the extract in different ingredients having several antibacterial modes of action. From our investigations on fractions F2 and F4 belonging to the fruits' crude extract, four compounds were characterizedSanjoinenine 1, betulinic acid 3 and epicatechin 4.

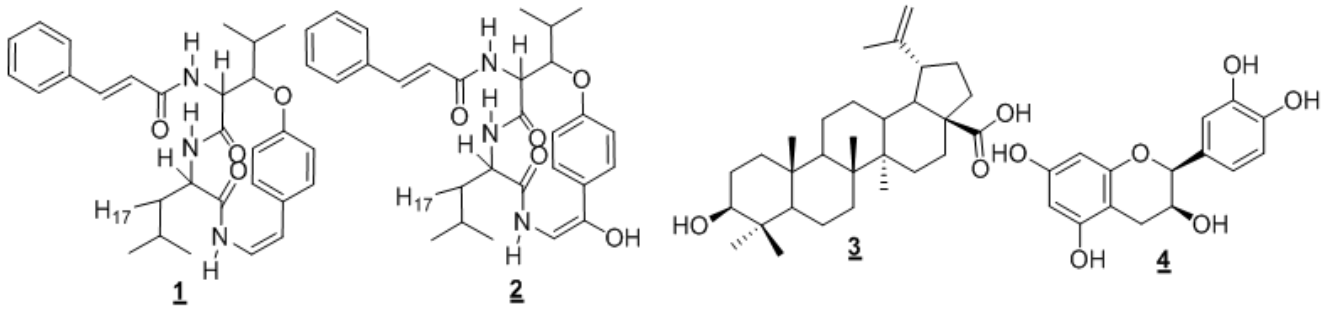

Figure 1. *Characterized Sanjoinenine 1, betulinic acid 3 and epicatechin 4.*

3.1. Identification of Sanjoinenine 1

Soluble in pyridine, compound 1 precipitates in hexane/ethyl acetate (25%) as a white powder. It responds positively to the Potassium tetraiodomercurate test: Valser-Mayer reagent, characteristic of alkaloids.

In its ^{13}C NMR spectrum (150 MHz, Pyridine-d5), we observe:

1) Three (03) signals attributable to the amide carbonyls and appearing at δ_C 172.5 (C-7); 168.9 (C-4) and 165.2 (C-22); two of which participate in the formation of the cyclopeptide and the other exocyclic respectively.
2) Four (04) signals corresponding to sp^2 hybridized carbons, respectively trans olefinic and ethylenic carbons at δ_C 140.9 (C-24); 130.6 (C-23) and 126.5 (C-2); 118.5 (C-1).
3) Two (02) signals attributable to carbons bound to heteroatoms, precisely to the oxygen atom and resonating at δ_C 156.8 (C-11) (bound to the aromatic ring) and 82.6 (C-9) (bound to an isopropyl group).
4) Signals from the aromatic region including two isochron

signals attributable to the a', b' system of an aromatic ring and resonating at δ_C 127.8 and 129.1.

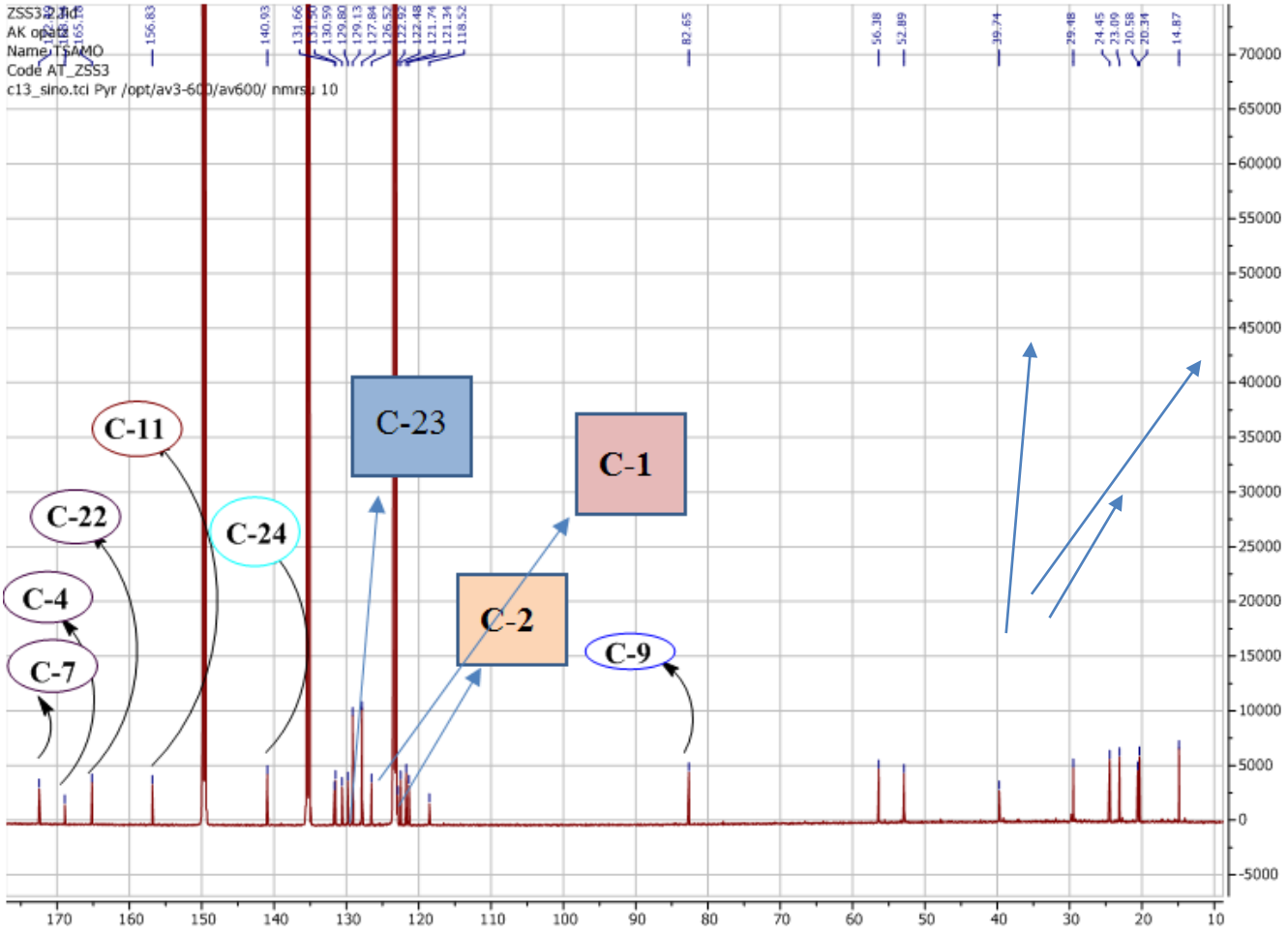

Figure 2. *^{13}C NMR spectrum (150 MHz, Pyridine-d_5) of compound 1.*

Its ^{1}H NMR spectrum (600 MHz, Pyridine-d_5, Figure 10), presents:

1) Four (04) integrative signals for one (01) proton each, attributable to the aromatic protons involved in the formation of the 14-membered ring. These protons resonate in the aromatic region at δ_H 7.04 (1H, d, H-12); 7.14 (1H, d, 6Hz, H-13); 6.97 (1H, dd, 6Hz, H-15); 7.44 (1H, dd, 6Hz, H-16); respectively. The mutiplicities of these are in agreement with those compared to the literature review and described by Abu-Zarga *et al*. [22].
2) Two (02) signals attributable to ethylenic protons belonging to a ring and always resonating in the strong fields at δ_H 6.49 (1H, t, H-1) and 6.93 (1H, t, H-2).
3) Three (03) signals, all appearing as a doublet and attributable to heteroatom protons, especially those bound to the nitrogen atom, resonating at δ_H 7.67 (1H, d, H-3); 9.20 (1H, d, H-6); 9.84 (1H, d, H-21) respectively.
4) One doublet and another doublet of doublet signal attributable to ethoxy and methoxy protons respectively appearing at δ_H 5.41 (1H, dd, (6; 0) Hz, H-8); 5.37 (1H, dd, (6; 0) Hz, H-9).
5) One (01) multiplet signal attributable to the proton bound to diastereoisotopic protons resonating at δ_H 4.69 (1H, m, H-5).
6) In the same ^{1}H NMR spectrum (600MHz, Pyridine-d_5, Figure 10), we observe:
7) Two (02) tripled signals, attributable to diastereoisotopic protons and resonant in weak fields at δ_H 1.59 (1H, dtd, H-17); 1.89 (1H, dtd, H-17') each.
8) Two (02) doublets of one (01) proton each attributable to the trans olefinic protons of the cinnanomoyl group and appearing in the strong fields at δ_H 7.07 (1H, d, H-23) and 8.11 (1H, d, H-24) respectively.
9) Two multiplet signals corresponding to the methine protons, resonating at δ_H 1.79 (1H, m*, H-18) and 2.47 (1H, m*, H-33) each.
10) Four (04) methyl doublets appearing in the weak fields at δ_H 0.65 (3H, d, H-19); 0.65 (3H, d, H-20); 1.20 (3H, d, H-32); 1.23 (3H, d, H-33), each coupling with a methine proton; suggesting the presence of two leucine units.

All these data allowed us to highlight:

The carbon skeleton of a 14-membered cyclopeptide alkaloid (A).

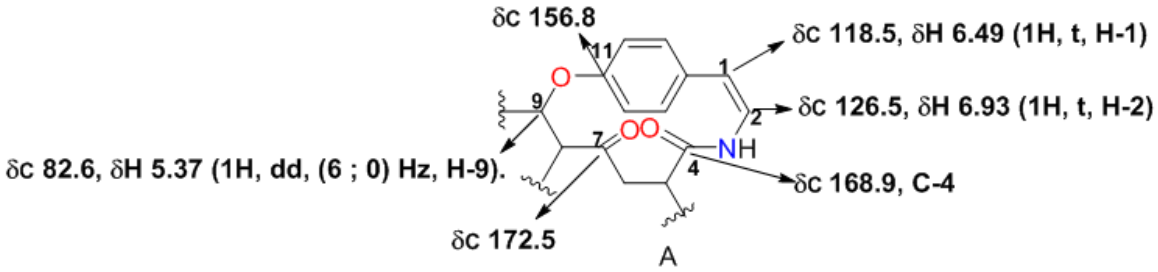

Figure 3. *The carbon skeleton of a 14-membered cyclopeptide alkaloid (A).*

The 3-phenylpropenamide

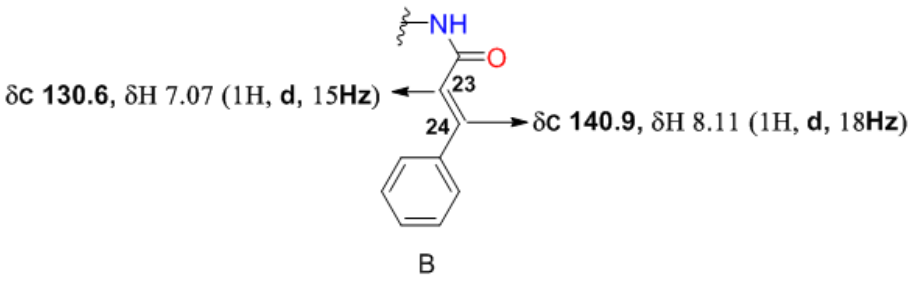

Figure 4. *The 3-phenylpropenamide.*

The Methylpropan

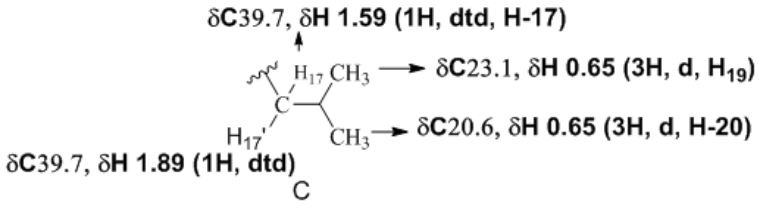

Figure 5. *The Methylpropan.*

The isopropyl group

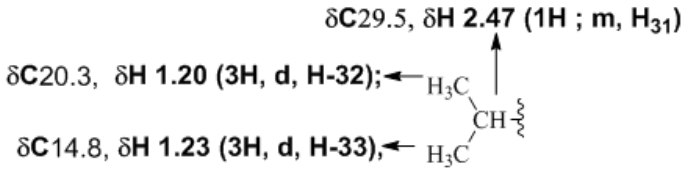

Figure 6. *The isopropyl group.*

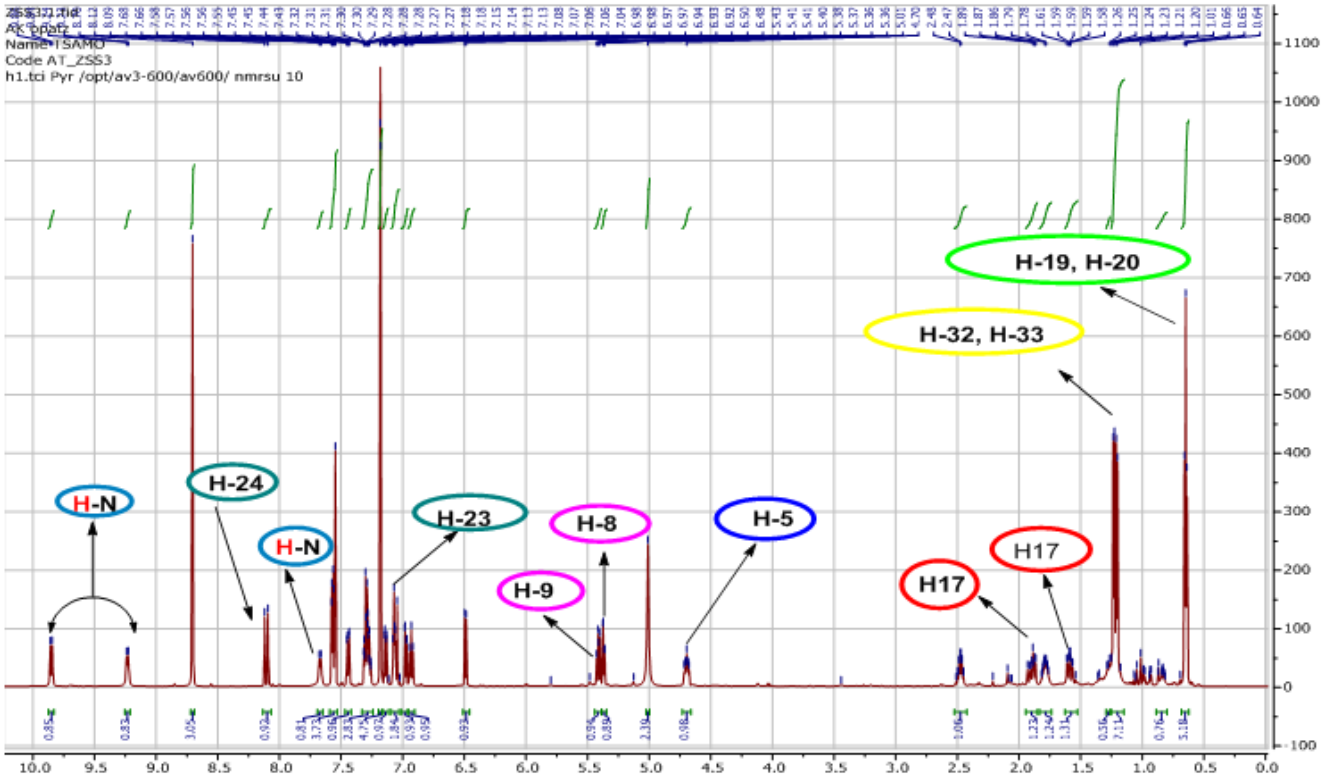

Figure 7. *^{1}H NMR spectrum (600 MHz, Pyridine-d_5) of compound 1.*

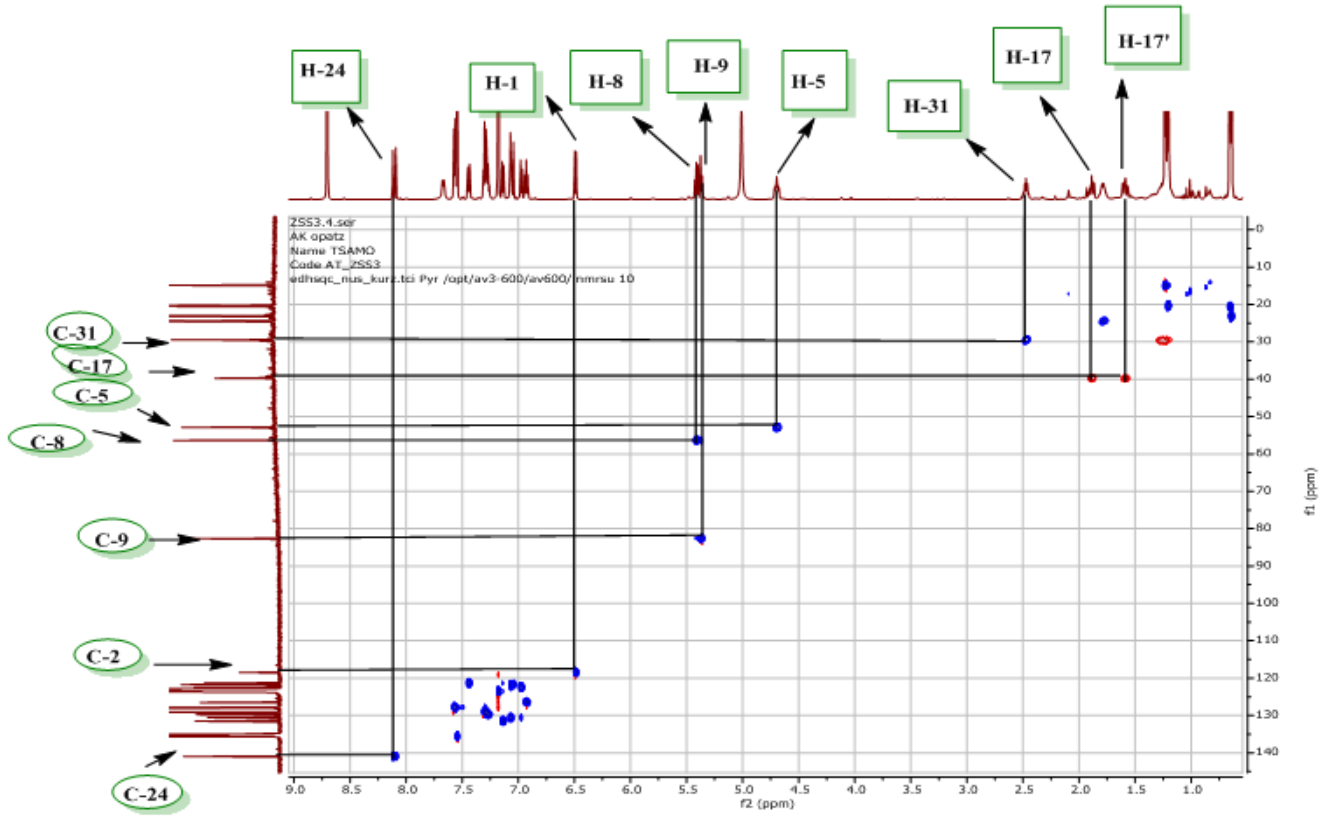

Figure 8. *HSQC spectrum of compound 1.*

3.2. Determination of the Positions of Groups B, C and D

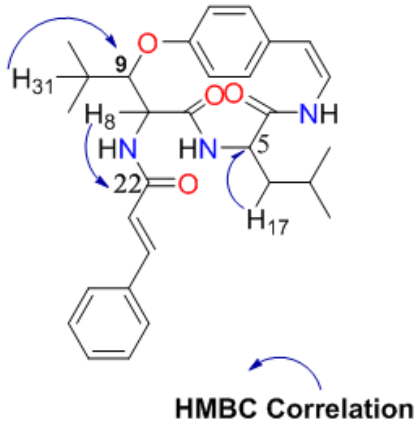

Figure 9. HMBC 1H-13C spectrum..

At this stage of the discussion, it will only be necessary to determine the position of the B, C and D groups on the carbon skeleton of a 14-membered cyclopeptide alkaloid.

This was made possible by the interpretation of its HMBC ^{1}H-^{13}C spectrum. Indeed, the correlation spots observed between the H-17 proton and C-5, H-8 and C-22, H-31 and C-9 allowed us to position the B, C and D groups at C-8, C-5 and C-9 respectively. This was corroborated by the correlation spots observed on the COSY ^{1}H-^{1}H spectrum between protons H-8 and H-21 on the one hand and between protons H-17 and H-5 on the other hand.

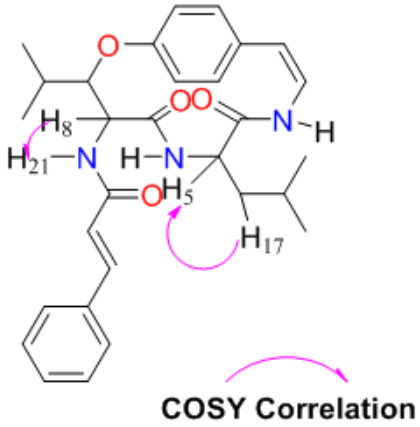

Figure 10. Correlations observed on the COSY spectrum.

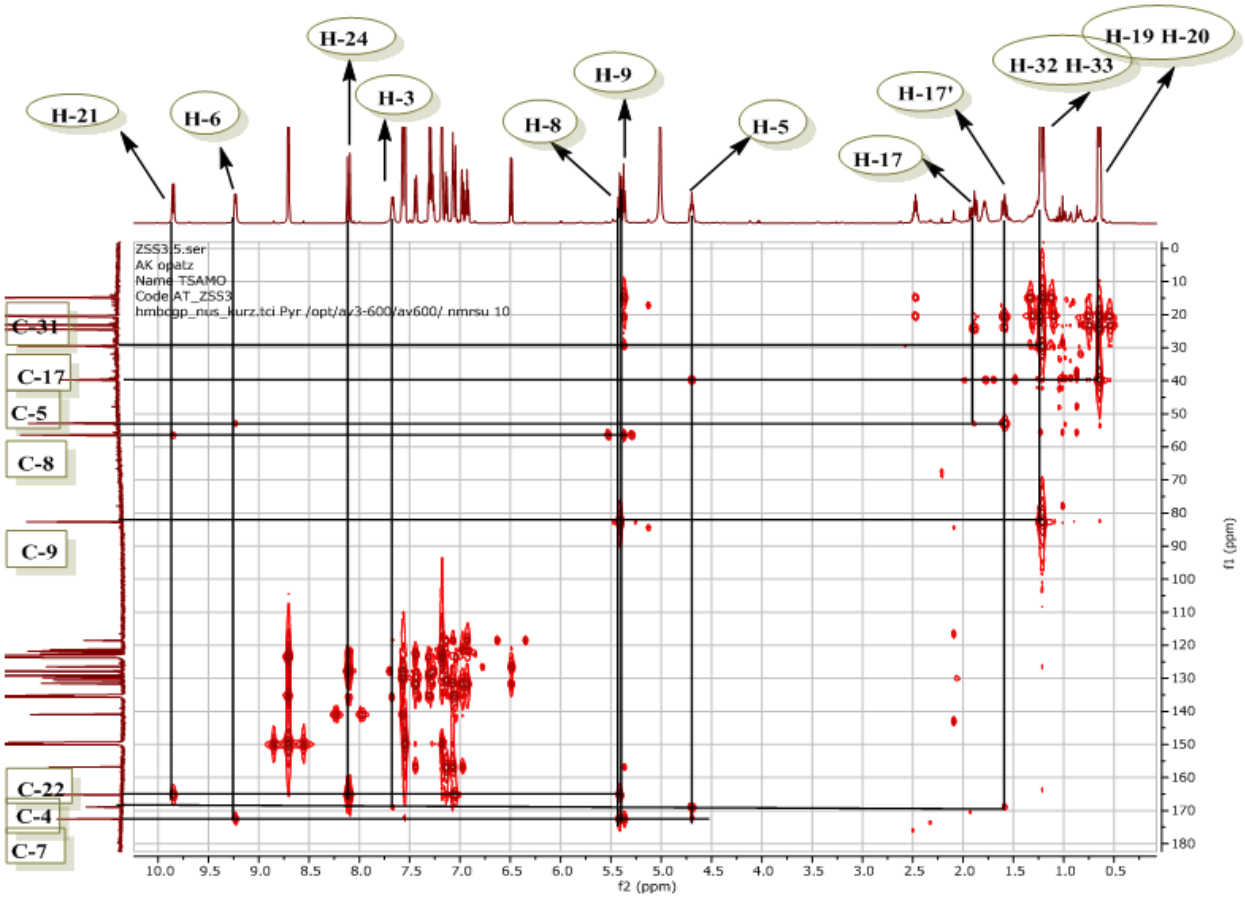

Figure 11. HMBC spectrum of compound 1.

All these spectroscopic data in agreement with those described by Tuenter E. *et al.*, [23]*;* allow us to identify the carbon skeleton of a 14-membered cyclopeptide alkaloid of the frangulanine type. To these, we add the description made by Han B. H. *et al.*, in 1982; and Abu-Zarga *et al.*, identifying the structure of an alkaloid cyclopeptide, isolated other times from the species *Ziziphus lotus* (Abu-Zarga *et al.*) and *Ziziphus vulgaris* [24], under the name of Sanjoinenine with the gross formula $C_{29}H_{35}N_3O_4$. The scheme 9 is showing the diferent correlations between the atoms.

Table 2. *Some Carbon-Proton correlations of compound 1 spectra comparing to that of the literature.*

Position	***δH* (ppm); nH; m; J (Hz)**	***δC* (ppm); (C5D5N)**	***δC* (ppm); Lit) (CDCl3)**
1.	7.07; 1H; d; 6	118.5	130.0
2.	6.93; 1H; t	126.5	132.7
3.	-	---	
4.	-	168.9	165.6
5.	4.69; 1H; m*	52.9	52.9
6.	-	---	
7.	-	172.5	171.9
8.	5.41; 1H; d; 6	56.4	55.6
9.	5.37; 1H; dd; 6-0	82.6	81.6
10.	-	---	
11.	-	156.8	156.0
12.	7.04; 1H; d; 6	121.7	118.8
13.	7.14; 1H; d; 6	131.5	123.0
14.	-	131.6	131.6
15.	6.97; 1H; dd; 6-0	122.5	125.7
16.	7.44; 1H; dd; 6-0	121.4	122.3
17.	1.89; 1.59; 2H; dtd	39.7	40.0
18.	1.79; 1H; m*	24.4	24.5
19.	0.65; 3H; d; 6	23.1	23.2
20.	0.65; 3H; d; 6	20.6	20.6
21.	-	---	---
22.	-	165.2	
23.	6.49; 1H; d; 12	130.6	127.8
24.	8.11; 1H; d; 18	140.9	143.3
25.	-	122.9	
26.	7.56; 1H; d, 6	127.8	127.8
27.	7.29; 1H; dd, 6-0	129.1	130.4
28.	7.29; 1H; dd, 6-0	129.8	127.9
29.	7.29; 1H; dd, 6-0	129.1	130.4
30.	7.56; 1H; d, 6	127.8	127.8
31.	2.47; 1H; m*	29.5	29.5
32.	1.20; 3H; d; 6	20.3	20.0

Position	*δH* (ppm); nH; m; J (Hz)	*δC* (ppm); (C5D5N)	*δC* (ppm); Lit) (CDCl3)
33.	1.23; 3H; d; 6	14.8	14.7

4. Conclusion

This study isolated and characterized secondary metabolites from Ziziphus mauritiana fruit and evaluated their antibacterial potential against Staphylococcus aureus, including reference strains and a clinical isolate. The work demonstrated that the crude extract (ZSA), as well as certain fractions, notably the alkaloid fraction F3, exhibited significant anti-staphylococcal activity, with minimum inhibitory concentrations (MICs) reaching up to 15.6 µg/mL against the S. aureus strain ATCC 25923. The activity of the crude extract, superior to that of some isolated fractions, suggests a promising synergistic effect between the different fruit constituents.

Among the identified compounds, sanjoinenin (1), betulinic acid (3), and epicatechin (4) were fully characterized by advanced spectroscopic methods (1D and 2D NMR). These molecules, belonging to the classes of cyclopeptide alkaloids, triterpenes, and flavonoids, are known for their diverse biological properties and could contribute to the observed activity.

These results validate the traditional use of Ziziphus mauritiana in the treatment of bacterial infections and highlight its potential as a source of compounds active against S. aureus, a priority pathogen due to the increasing prevalence of resistant strains, including MRSA. The promising MICs, particularly of the crude extract, warrant further investigation to isolate other active constituents, evaluate their mechanisms of action, and study their in vivo efficacy as well as their potential toxicity. This research thus paves the way for the development of new natural antimicrobial agents or complementary phytomedicines in the fight against staphylococcal infections.

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Conflicts of Interest

The authors declare no conflicts of interest.

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