

HPLC Analysis and Antimicrobial Screening of Methanol Extract/Fractions of the Root of *Millettia aboensis* (Hook.f.) Baker against *Streptococcus mutans*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors UCE, EEA, AJI and AJD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors UCE, EEA, AJD, FNE, AJI, ALO, FON, JNE, ESI, NUB, JON, OOK and AAT managed the analyses of the study. Authors EEA and AJI managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2020/v39i2230835

Editor(s):

(1) Dr. Yahya Elshimali, Charles R. Drew University of Medicine and Science, USA.

Reviewers:

(1) Evelyn Sharon Sukumaran, SRM College of Pharmacy, India.

(2) Monika Laxman Jadhav, SNDT Women's University, India.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/59930>

Original Research Article

Received 29 May 2020
Accepted 05 August 2020
Published 10 August 2020

ABSTRACT

Millettia aboensis (Hook.f.) Baker belongs to the Leguminosae family, known locally as nduezi in Igbo, erurumesi in Edo, and Ôdúdū in Efik. The plant parts have proven to be active against some diseases. The plant was studied for its antimicrobial activity and the phytochemicals present. The plant materials were macerated and fractionated using different extraction methods. The compounds present in the fractions of the stem were detected with the aid of HPLC-DAD. The efficacy of the crude methanol and fractions from the root part was evaluated against *Streptococcus mutans*. The results showed that the aqueous fraction extract had the highest percentage yield

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(51.11%), followed by the ethyl acetate (24.10%), butanol (6.63%), and hexane (5.56%), while the methanol extract had a yield of 2.48%. Nine phytochemical compounds were detected from the fractions of the root extract: bis-oxazolidinone derivative – 1, circumdatin F – 2, enniatin B – 3, septicine – 4, orobol 8-C-glucoside – 5, rocaglamide – 6, genistein 6-C-glucoside – 7, 3-phenyl chromen-4-one – 8, and corynesidone D – 9. The methanol extract/fractions of *M. aboensis* showed no antimicrobial activities on clinical isolates at different concentrations (6.25-50 mg/ml). Based on the poor activity against *Strep. mutans*, but with promising phytoconstituents present, the extract/fractions need further investigation as regards its utilization against other pathogens.

Keywords: *Millettia aboensis*; *Streptococcus mutans*; HPLC; dental caries; secondary metabolites; antibacterial.

1. INTRODUCTION

Infectious diseases are disorders caused by infectious agents such as bacteria, viruses, fungi or, parasites and, poses a great burden on many societies [1]. These infectious agents are capable of causing chronic diseases that affect the health of individuals and the globe [2]. Plants that have been utilized for centuries to alleviate infectious diseases are considered as great significant information for newer antimicrobial candidates [3].

Millettia aboensis (Hook.f.) Baker is a plant belonging to the Leguminosae family commonly known as nduezi in Igbo, erurumesi in Edo, and Òdúdū in Efik. Almost all the parts of *M. aboensis* have medicinal properties. The leaf is used by traditional herbalists for general healing including ulcer healing and laxatives, while the root is used in the treatment of gastrointestinal disturbances and liver diseases. Also, the leaf, stem, and root mixed with other plant materials (herbs) are used to cure venereal diseases such as gonorrhoea, syphilis, etc. [4,5,6]. Onyegeme-Okerenta and Okafor [6] reported on antimicrobial properties of ethanol leaf extract of *M. aboensis* on some selected clinical isolates such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The report showed promising antimicrobial activities against these agents.

The general purpose of this research work was to determine the antimicrobial abilities of the crude extract and fractions of the root of *M. aboensis* against *S. mutans* and to validate the medicinal importance of the plant extract and fractions.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

The root part of *M. aboensis* was collected from Amudo-Awka, Anambra State of Nigeria, the

plant was identified by Mr. Alfred Ozieko of Bioresource Development and Conservation Program (BDCP), Nsukka. A specimen was deposited in the herbarium section of the Department Of Pharmacognosy And Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, with specimen number PCG/474/A/021. The impurities from the dried root of the plant were removed and grounded into a fine powder using a mechanical grinder. Powder material was maintained at room temperature at about 25-27°C.

2.2 Crude Extraction and Fractions Preparation

800 g of free from moisture powder of the root of *M. aboensis* was weighed and extracted for 48 hours using cold maceration method with periodic shaking. The maceration steps were carried out once in methanol for complete extraction. The methanol extract was collected and then concentrated to dryness under vacuum at 40°C using a rotary evaporator. The concentrated methanol root extract (MRE) was reconstituted with 200 ml of methanol: water (2:8) and fractionated successively with hexane, ethyl acetate and butanol to give the hexane (MRHF), ethyl acetate (MREF), butanol (MRBF) and aqueous (MRWF) fractions. The fractions so obtained were filtered twice using Whatman no. 1 filter paper. The rotary evaporator was utilized in concentrating the fractions at 45±5°C. The extracts of the plant were analyzed for their antimicrobial property.

2.3 Preparation of Test Organisms

Clinical isolates of *Streptococcus mutans* were obtained from carious lesions of patients that attended the clinic at Federal College of Dental Therapy and Technology, Trans Ekulu, Enugu. All reagents used for the studies were prepared and stored in a refrigerator. Subsequently, brain heart blood agar and infusion broth were prepared and autoclaved at 121°C for 15

minutes. 20 ml of blood agar and brain infusion broth were dispensed into sterile bijoux bottles and Petri dishes respectively. Sterile bijoux bottles holding 20 ml of brain infusion were taken down to the surgery rooms. The tooth of patients that presented with a carious lesion was extracted by the dental surgeon under strict aseptic condition. Teeth extracted by the dental surgeon were introduced into sterile bijoux bottles holding 20 ml of brain heart infusion broth and labelled accordingly. After 5 hours sterile swab sticks were inserted into the bijoux bottles containing the extracted tooth and they were spread onto solid blood agar plates. The plates were developed at 37°C for 48 hours aerobically and at 37°C for 72 hours anaerobically using a gas pack for each patient sample [7]. Colonies that grew were respectively subcultured into prepared blood agar using a streaking method for isolation of pure colonies. The plates were incubated aerobically and anaerobic culture conditions. Pure colonies were identified by macroscopic examination of colony growth on agar plates, morphological characteristics, motility tests, and other biochemical tests according to standard microbiological procedures [7]. All the organisms that were isolated and identified were stabbed in nutrient agar slant, labeled appropriately, and kept in the refrigerator.

2.4 Antimicrobial Assay

2.4.1 Primary antimicrobial screening

Primary screening of the plant extracts for antibacterial activity was carried out using the agar well diffusion method as previously described by Onyegbule *et al.*, [8]. Dilutions of 50, 25, 12.5, and 6.25 mg/mL in DMSO (100% v/v) were prepared for each of the plant extract in a 2-fold dilution process. 20 mL of molten Mueller-Hinton Agar was poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates, and holes (6 mm) were made in the agar plates using a sterile metal cork-borer. 20 µl of the various dilutions of the extract and controls were put in each hole under aseptic conditions. This was kept at room temperature for 1 h to allow the agents to diffuse into the agar medium and incubated accordingly. Gentamicin (10 µg/mL) was used as positive controls in the antibacterial evaluation; while DMSO (100% v/v) was used as the negative control. The Mueller-Hinton Agar plates were then incubated at 37°C for 24 h. The resulting inhibition zones diameters (IZDs) were measured and recorded. The size of

the cork borer (6 mm) was deducted from the values recorded for the IZDs to obtain the actual zone diameters. This procedure was conducted in duplicate, and the mean IZDs calculated.

2.4.2 Analytical HPLC of the fractions

This was carried out according to the method of Ajaegbu *et al.*, [9]. Each of the dried fractions was reconstituted with 2 ml of HPLC grade methanol. 100 µl of the dissolved samples were each transferred into High performance liquid chromatography (HPLC) vials containing 500 µl of HPLC grade methanol. HPLC analysis of the samples was carried with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, DionexSoftron GmbH, Germering, Germany). Detection was at 235, 254, 280, and 340 nm. The separation column (125 x 0.4 cm; length x internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The absorption peaks for the plant extract were analyzed by comparing it with those in the HPLC-UV/Visible library.

3. RESULTS

3.1 Percentage Yield

The results of the percentage yield of the extract and fractions of the extraction process of *M. aboensis* root are shown in Table 1. The results showed that aqueous fraction had the highest percentage yield (51.11%), followed by ethyl acetate (24.10%), butanol (6.63%), hexane (5.56%), while methanol extract had a yield of 2.48%.

Table 1. Percentage Yield of the extract and fractions

Extracts	Yield (% w/w)
Methanol ^a	2.48
Hexane fraction ^b	5.56
Ethyl acetate fraction ^b	24.10
Butanol fraction ^b	6.63
Water fraction ^b	51.11

^a = yield calculated from 800 g of plant material ^b = yield calculated from 19.84 g of methanol root extract

3.2 HPLC-DAD Analysis

The HPLC analysis of the fractions of the root of *M. aboensis* was suggestive of the presence of nine phytochemicals. The compounds detected

were: bis-oxazolidinone derivative – 1, circumdatin F – 2, enniatin B – 3, septicine – 4, orobol 8-C-glucoside – 5, rocaglamide – 6, genistein 6-C-glucoside – 7, 3-phenyl chromen-

4-one – 8, and corynesidone D – 9 are shown in Figs. 1 – 4. The retention time, λ -max, type of phytochemical, molecular weight, and formulae are shown in Table 2.

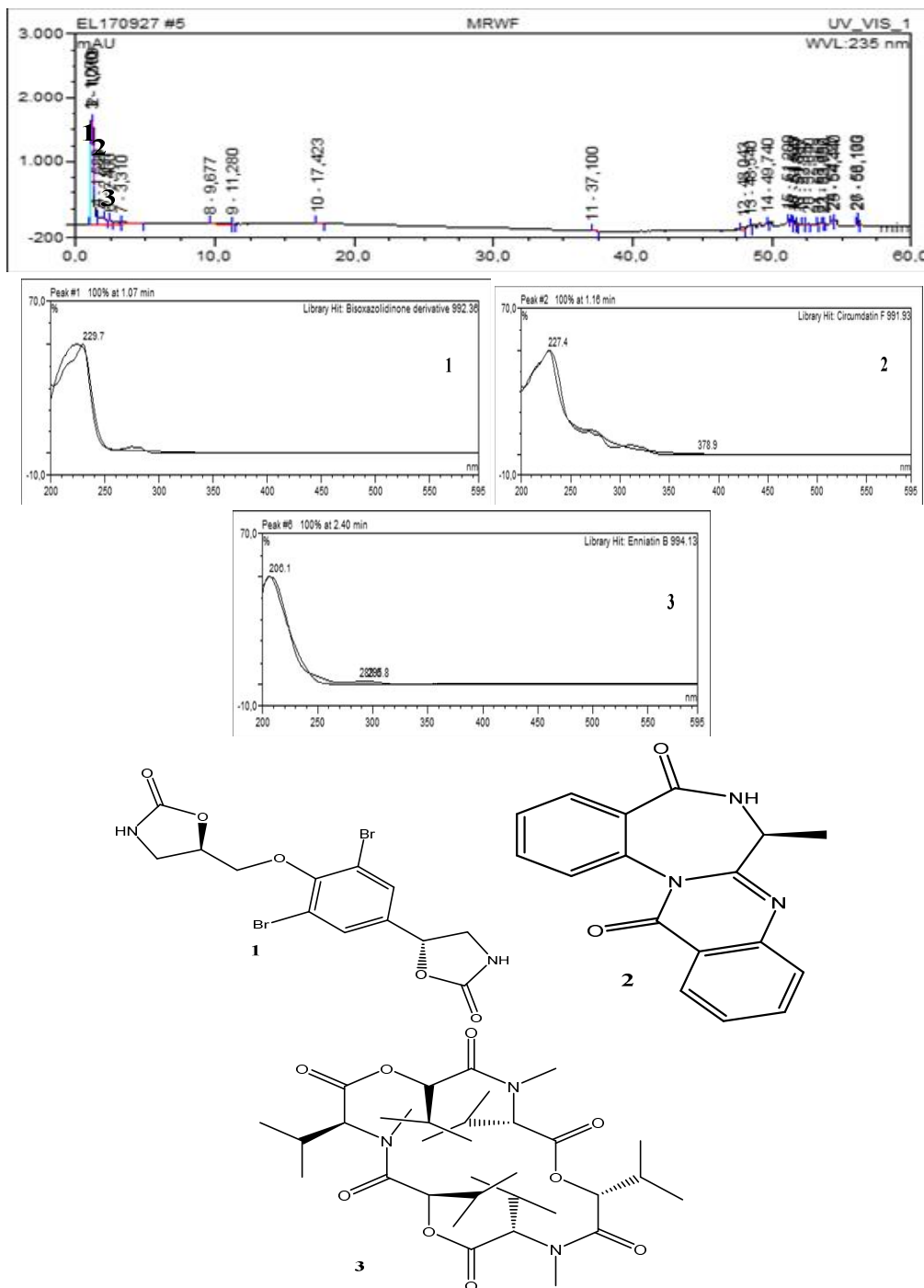


Fig. 1. HPLC chromatogram of MRWF showing bis-oxazolidinone derivative, circumdatin F, and enniatin B; their UV spectra; and structures

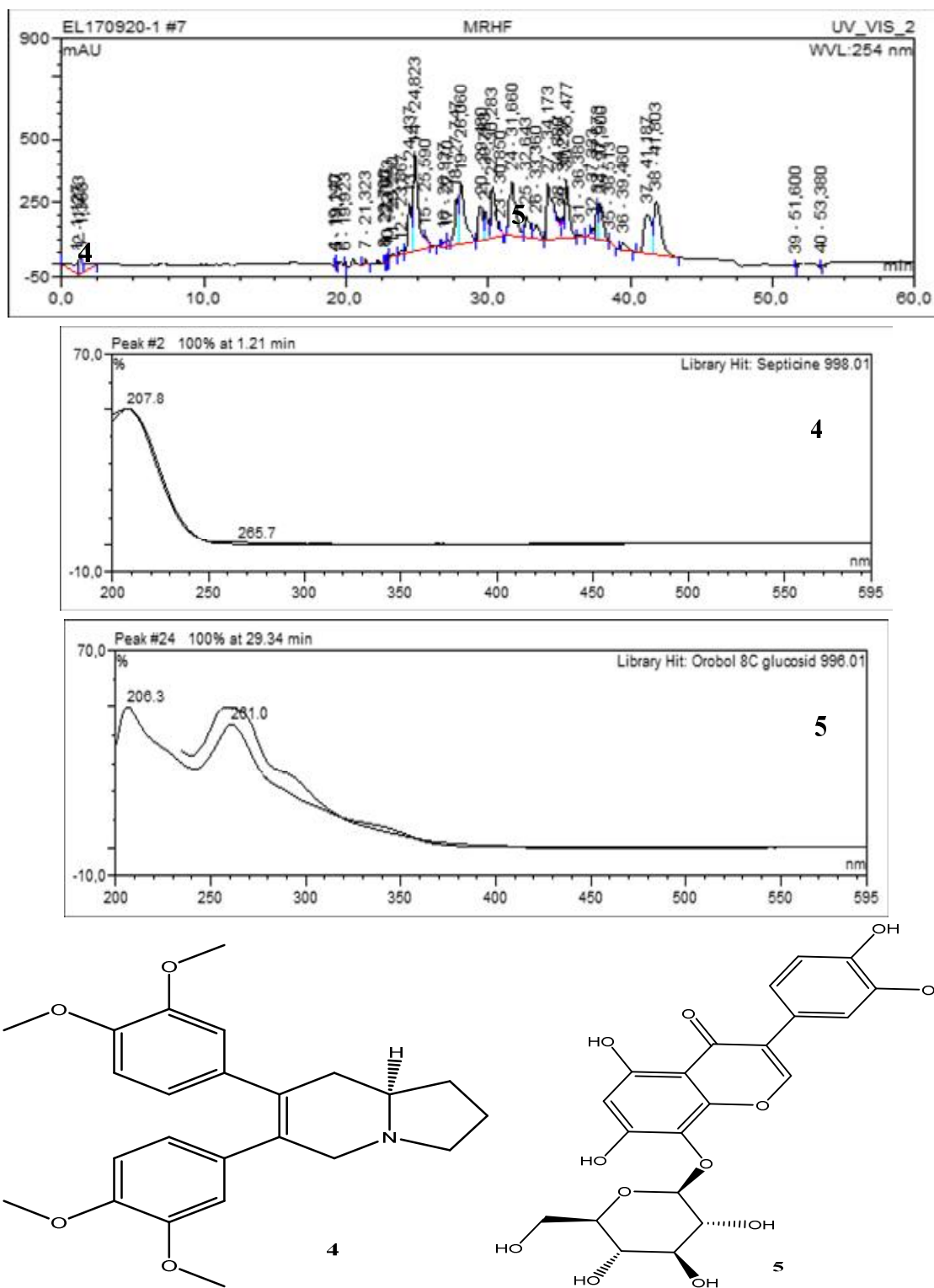


Fig. 2. HPLC chromatogram of MRHF showing septicine and orobol 8-C-glucoside; their UV spectra; and structures

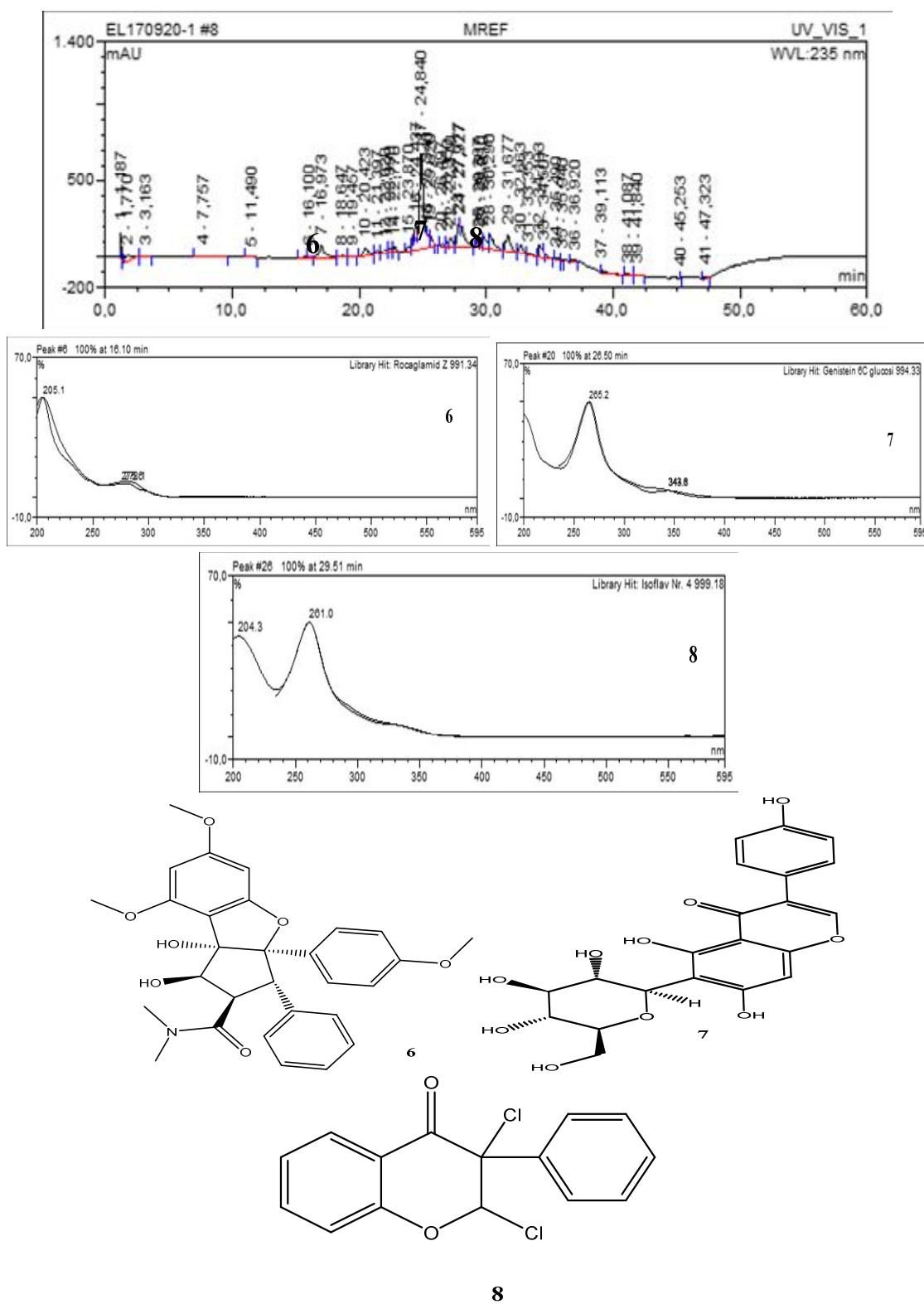


Fig. 3. HPLC chromatogram of MREF showing rocaglamide, genistein 6-C-glucoside, and 3-phenyl chromen-4-one; their UV spectra; and structures

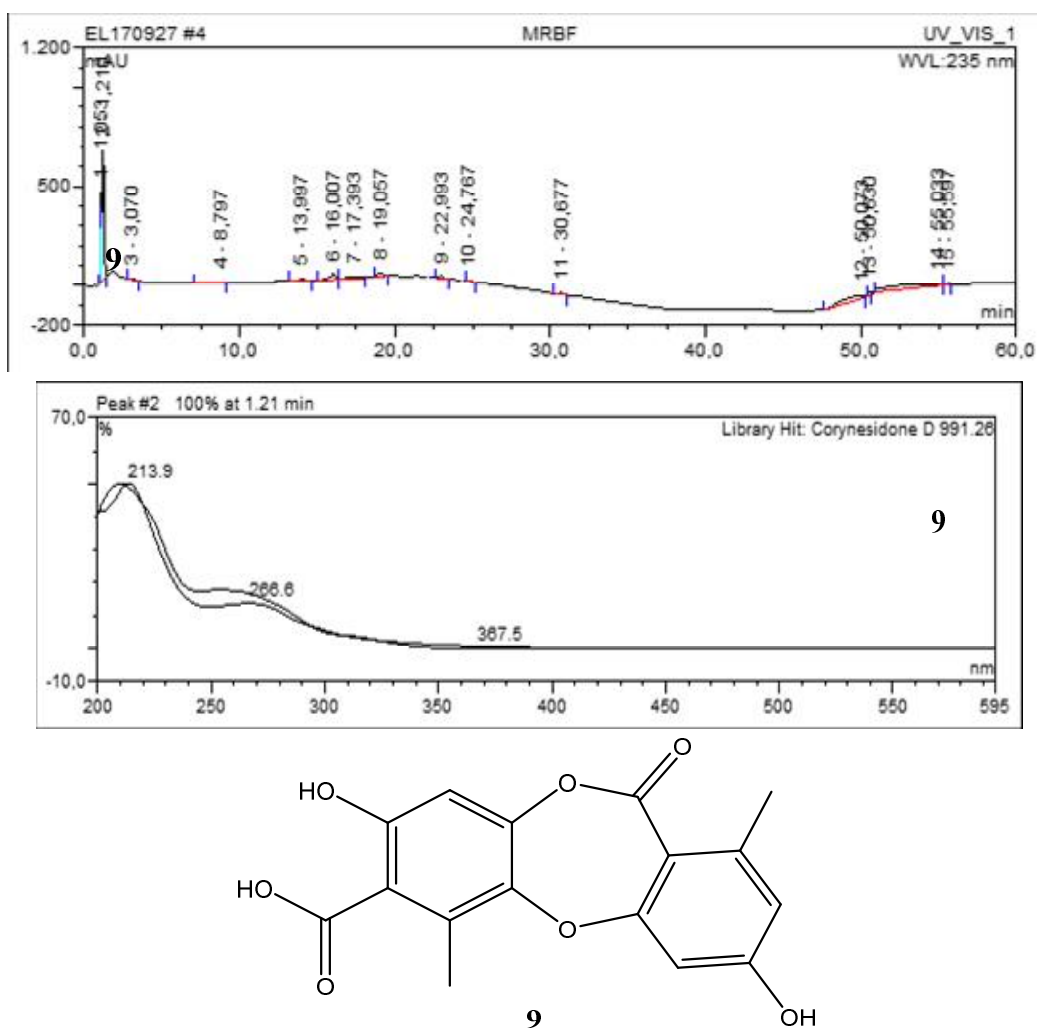


Fig. 4. HPLC chromatogram of MRBF showing corynesidone D; its UV spectrum; and structure

3.3 Antimicrobial Ability

The results of the antimicrobial ability are presented in Table 3. The methanol crude extract and fractions of the root of *M. aboensis* showed no inhibitory activity against *S. mutans*.

4. DISCUSSION

The result of this study showed that aqueous fraction had the highest percentage yield (51.11%), followed by ethyl acetate (24.10%), butanol (6.63%), hexane (5.56%), while methanol extract had a yield of 2.48%. This is in line with the research conducted on phytochemical analyses of ethanol and water extracts Of *M. aboensis*, *Cuscuta reflexa*,

Daniella oliveri and *Synclisia scabrida* by Adonu et al., [5]; he reported that the percentage yields of all the water extracts are greater than those of the ethanol extracts. The yields of the root extracts of *M. aboensis* are the greatest compared with all other extracts.

The result showed the presence of nine compounds in all the fractions of aqueous, hexane, ethyl acetate, and butanol when serially subjected to HPLC-DAD analysis. Nine phytochemical compounds were detected: bisoxazolidinone derivative – 1, circumdatin F – 2, enniatin B – 3, septicine – 4, orobol 8-C-glucoside – 5, rocaglamide – 6, genistein 6-C-glucoside – 7, 3-phenyl chromen-4-one – 8, and corynesidone D – 9 as shown in Table 2 and Figs.1 – 4.

Table 2. The wavelength for maximum absorbance λ_{\max} and retention time of the phytochemical compounds detected by HPLC-DADS for all the fractions of methanol extract

Fraction	Compound	Class of Phytochemical	Molecular Formulae	Molecular Weight	Rt (min)	λ_{\max} (nm)
MRWF	Bis-oxazolidinone	Alkaloid	C ₁₃ H ₁₂ Br ₂ N ₂ O ₅	436.06 g/mol	1.07	229.7
MRWF	Circumdatin F	Alkaloid	C ₁₇ H ₁₃ N ₃ O ₂	291.3 g/mol	1.16	227.4, 378.9
MRWF	Enniatin B	Mycotoxin	C ₃₃ H ₅₇ N ₃ O ₉	639.8 g/mol	2.40	206.1, 296.8
MRHF	Septicine	Alkaloid	C ₂₄ H ₂₉ NO ₄	395.5 g/mol	1.21	2207.8, 265.7
MRHF	Orobol 8-C-glucoside	Flavonoid	C ₂₁ H ₂₀ O ₁₁	448.38 g/mol	29.34	2206.3, 261.0
MREF	Rocaglamide	Alkaloid	C ₂₉ H ₃₁ NO ₇	505.6 g/mol	16.10	205.1, 286.1
MREF	Genistein 6-C-glucoside	Flavonoid	C ₂₁ H ₂₀ O ₁₀	432.4 g/mol	26.50	285.2, 343.8
MREF	3-phenyl chromen-4-one	Flavonoid	C ₁₅ H ₁₀ O ₂	222.24 g/mol	29.51	204.3, 261.0
MRBF	Corynesidone D	Mycotoxin	C ₁₆ H ₁₂ O ₇	316.26 g/mol	1.21	213.9, 206.6, 362.5

MRWF= Methanol root aqueous fraction, MRHF= Methanol root hexane fraction, MREF= Methanol root ethyl acetate fraction, MRBF= Methanol root butanol fraction

Table 3. Zone of inhibition in mm produced by the crude extract/fractions of *M. aboensis* root

Sample	Concentration (mg/ml)			
	6.25	12.5	25	50
MRE	0	0	0	0
MRHF	0	0	0	0
MREF	0	0	0	0
MRBF	0	0	0	0
MRWF	0	0	0	0
Positive control Gentamicin (10 µg ml)	-	-	-	25
Negative control DMSO	0	0	0	0

MRHF = Methanol root hexane fraction, MREF = Methanol root ethyl acetate fraction, MRBF = Methanol root butanol fraction, MRWF = Methanol root aqueous fraction

Bis-oxazolidinone derivatives – 1 have been reported to possess anti-bacterial, and anti-tubercular activities [10,11,12]. It catalyzes the process of peptide bond formation due to the peptidyl transferase center that is the active site of oxazolidinones in the ribosome [13] and the presence of this compound in the plant may have medicinal importance in drug discovery.

Circumdatin F–2 has IUPAC nomenclature 7-methyl-6,7-dihydroquinazolino[3,2-a][[1,5]benzodiazepin-13(5H)-one and this was reported to possess antiepileptic, tranquilizers and effective as non-nucleoside inhibitors of HIV-1 reverse transcriptase [14].

Enniatin B–3 has been reported to exhibit varieties of pharmacological activities including antimycobacterial, antihelmintic, antifungal, herbicidal, and insecticidal activities. They inhibit various enzymes, for example, acyl CoA–cholesterol–acyltransferase and cyclic nucleotide phosphodiesterase [15,16,17].

Septicine – 4 has been reported to possess anti-inflammatory and anticancer properties [18].

Orobol 8-C-glucoside – 5 was investigated regarding its potential immune-modulating effect and has pharmacological action [19,20].

Rocaglamide – 6 has been reported to exhibit a variety of pharmacological activities including anti-inflammatory, inhibition of protein synthesis, reduce Eμ-Myc driven lymphomas, *eukaryotic translation inhibition activity*, and insecticidal [21,22,23,24,25].

Genistein 6-C-glucoside – 7 was revealed to have the potency of a compound as an anti-cancer and provide protection against disorders of the cardiovascular system [26,27,28].

3-phenyl chromen-4-one or [3-phenyl-1,4-benzopyrone] structure – 8 is an isoflavonoid compound. The compound has been reported to exhibit anti-oxidants and assist in the prevention of cancer and heart disease [29].

Corynesidone D – 9 was revealed to possess antimicrobial, antioxidant, and anticancer activities [30,31]. All these nine compounds have biological activities.

It was observed in this study that methanol extract/fractions of *M. aboensis* showed no antimicrobial activities against *S. mutans* at

different concentrations (6.25 – 50 mg/ml) – Table 3 despite the presence of nine phytochemical compounds in the fractions of the methanol extract. This reason could be as a result the MIC was not enough to cause inhibitory growth to the tested organism or the root of the plant has no potency against *S. mutans*.

5. CONCLUSION

From this study, HPLC-DAD techniques revealed the presence of nine phytochemical compounds and it can be concluded that the antimicrobial screening of the methanol extract/ fractions of *M. aboensis* showed no activity against *S. mutans*, but with promising phytoconstituents present, the extract/fractions need further investigation in its utilization against other pathogens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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