



## **Antimicrobial Evaluation of the Extract/Fractions of the *Millettia aboensis* (Leguminosae) Stem against *Streptococcus mutans***

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Authors UCE, EEA and AJD conceptualize the work. Authors UCE, EEA, AJD, FNE and ALO done the methodology. Authors UCE, EEA, AJD, FNE, AJI, ALO, FON, JNE, ESI and AAT completed the investigation. Authors UCE, EEA, AJD, FNE, AJI, ALO, FON, JNE, ESI, NUB and AAT done the formal analysis. Authors UCE, EEA, AJD, FNE and ALO completed the validation. Author EEA completed the data curation. Authors FNE and ALO done the writing and original draft preparation. Author EEA reviewed and edited the writing. Author EEA managed the overall supervision. Author EEA done the project administration. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** *Millettia aboensis* (Hook.f.) Baker belongs to the Leguminosae family, known locally as nduezi in Igbo, erurumesi in Edo, and Òdúdū in Efik. *Millettia aboensis* stem is a rich source of flavonoids, phenolic acid, alkaloids, and steroids, hence with medicinal and physiological potentials. It is used in traditional medicine for general healing of diseases including ulcers and laxatives. The present study was aimed at evaluating the antibacterial potential of the extract/fractions of the stem of *M. aboensis* against *Streptococcus mutans* – dental caries causative organism and detection of its principles.

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**Methodology:** Cold maceration in methanol and liquid-liquid fractionation techniques using hexane, ethyl acetate, and butanol as solvents were utilized for the extraction and fractionation processes respectively. Some phytochemicals from the fractions were suspected using High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD). The crude methanol stem extract/fractions were screened to analyze its antibacterial activity against *Streptococcus mutans*.

**Results:** The results showed for the fractions that aqueous fraction extract had the highest percentage yield (57.73%), followed by ethyl acetate (16.79%), butanol (12.17%), hexane (9.48%), while the percentage yield of the methanol extract is 2.98%. The HPLC-DAD analysis detected the following phytochemical constituents from the stem fractions: pestalotioprolide C - 1, corynesidone D - 2, enniatin B - 3, dipiperamide E - 4, isopranetin 8-C-glucoside - 5, genistein 8-C-glucoside - 6, genistein 6-C-glucoside - 7, and peniciaculin B - 8. In relatively low and high concentrations (6.25 – 50 mg/ml), the extract/fractions of *M. aboensis* were found ineffective against *Streptococcus mutans*.

**Conclusion:** The plant – *M. aboensis* with its phytochemicals present could be an excellent source of novel biologically active compounds with pharmaceutical and industrial importance.

**Keywords:** *Millettia aboensis*; *streptococcus mutans*; antibacterial; HPLC-DAD; dental caries.

## 1. INTRODUCTION

Traditional medicine, being the preferred basic health care system in many rural communities, is a medical practice where extracts from plants or the different parts of the plants are used as a source of therapeutic and curative aid [1]. These plants are known as medicinal plants and they play vital roles in human health [2]. Chen et al. [3] stated that the use of medicinal plants has increased worldwide due to the high demand for herbal drugs, natural health products, and secondary metabolites of the medicinal plants. Medicinal plant as Ezekwesili-Ofili and Okaka [4] described, is any plant, either whole or parts, that can be used for a therapeutic purpose or as a precursor for drug synthesis. They are also referred to as “unorganized drugs” if the extracts are from the oil, gums gels exudates, and balsams or “organized drug” if their extracts are from the stem, back root, and leaf. Medicinal plants as Onyeregeme-Okerenta and Okafor [5], reported, play a vital role in the treatment of multiple diseases and infections and it is also biodegradable and readily available not to mention being cost effective. It is also without side effects and is eco-friendly. According to Mintah et al., [1] over 80% of the world's population depends on medicinal plants because the active compounds isolated from these plants are used in modern medicine for drug production. Given this fact, a variety of drugs would be best obtained from medicinal plants [6] because of their secondary metabolites which include phenolic compounds, alkaloids, flavonoids, and tannins [7]. The secondary metabolites from medicinal plants are obtained in different

concentrations for different plants or different parts of a plant, thereby giving each plant a unique medical property [8]. These metabolites work individually or together to reduce the undesired adverse side effect to the nearest minimum and stabilize the active compounds or the phytochemicals of the plant. The combined effort surpasses their action individually thereby increasing or decreasing the assimilation of the medical constituents into the body [9]. Some medicinal plants are also known for their antimicrobial effects against pathogenic microorganisms [8]. Atikya et al., [8] in their work revealed that the rapid increase in the interest of medicinal plants was due to their antioxidant activities, cost effectiveness, and low toxicity, but the important is their antibacterial effect which is engendered by the increased resistivity in microorganisms. Due to the high rate of microbial infection and the rate by which microorganisms become resistant to antibiotics, there is an urgent need to discover and develop new and potent antimicrobial agents to enhance the health of mankind [10,11]. Judging from records, the new families of antimicrobial agent will stand only for a short period with the widespread emergence of resistivity [12,13]. *Millettia aboensis* as Nduka et al., [14] reported is a multipurpose medicine. It is a small tree of 30-40 feet high with a rust-hairy leaves and purple flowers, from the family of Leguminosae, popularly known as “otoroekpo or uturuekpa” in some parts of Igbo land in Nigeria. It is commonly found in the low land rain forests and characterised by dark reddish wood [4,5]. Although it is effective for the treatment of constipation in children and other health issues such as cold and catarrh, diarrhoea, chickenpox,

dysentery, headache, and measles, it also has antimicrobial effects on pathogenic microorganisms [14,15,16]. Different parts of *Millettia aboensis* are specific to a particular infection or disease. Onyegeme-Okerenta and Okafor [5], reported the management of some sexually transmitted diseases using the leaf extract, but the stem extract used as a laxative for both children and adults. They also stated that a mixture of leaf, root, and stem are used for the treatment of venereal diseases. In this study, the antibacterial effects of the stem extracts are evaluated against the causative agent of dental caries – *Streptococcus mutans*.

## 2. METHODS

### 2.1 Sample Collection

*Millettia aboensis* was collected from its natural habitat in Amudo-Akwa, Anambara State of Nigeria in January 2015. Identification was done by Mr. Alfred Ozieko of Bioresources, Development, and Conservation Program (BDGP), Nsukka. The sample was deposited with the specimen number of PCG/474/A/021 at the herbarium section of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka. The sample was dried and using a mechanical grinder, it was grounded into a fine powder. The grounded sample was stored away from light at room temperature (25°C – 27°C) until required for extraction and analysis.

### 2.2 Extraction

This was carried out according to the method of Ajaegbu et al. [17] with some modifications. Exhaustive extraction was done with 900 g of the powdered stem sample for 2 days using the cold maceration method in methanol with intermittent shaking. To ensure maximum extraction, the process was repeated in methanol. The methanol extract was collected and concentrated under vacuum using a rotary evaporator to near dryness at 40°C. The concentrated stem extract (18.5 g) was restored to its original form by adding 200 ml of methanol and water at the ratio of 2:8. The solution was successively fractionated with hexane, ethyl acetate and butanol to give hexane fraction (MSHF), ethyl acetate fraction (MSEF), butanol fraction (MSBF), and aqueous fraction (MSWF).

### 2.3 Clinical Sample Collection and Inoculation

A total of 36 samples were collected from the carious lesion of patients at the Federal College of Dental Technology and Therapy, Trans Ekulu, Enugu. The teeth were extracted by the dental surgeon under strict aseptic condition and were immediately inoculated into sterile bijoux bottles containing 20 ml of freshly prepared brain heart infusion broth each and labelled accordingly. After 5 h, the specimen is subcultured into plates of freshly prepared blood agar using a sterile swab stick and incubated for aerobically and anaerobically at 37°C for 24 h and 72 h respectively. Pure cultures were isolated using the streaking method, labelled and incubated aerobically, and anaerobically. All pure isolates were store in nutrient agar slant, well labelled and refrigerated [18].

### 2.4 Bacteria Identification

#### 2.4.1 Physical tests

Physical identification was done by macroscopic examination of colonies, gram staining, and motility test. Gram staining technique was carried out according to the method of Cheesbrough [18]. Smears of the isolate were made on sterile glass slides and were heat fixed. The smear was stained with crystal violet for 1 min, washed with gentle running tap water, flooded with dilute gram's iodine solution for 1 min, washed with gently running tap water, decolorized with 95% alcohol for 30 sec, counterstained with safranin solution for 30 sec, washed with running water, air-dried and viewed under oil immersion objective lens.

A motility test was performed using the hanging drop technique as Cheesbrough [18] described. A little Vaseline jelly was rubbed around the cavity of hanging drop slide. A drop of peptone water containing the pure culture was placed on a coverslip. The hanging drop slide was then placed over the drop of peptone water in such a way that the depression lies over the drop. The slide is quickly inverted and viewed under the microscope using the X40 objective lens.

#### 2.4.2 Biochemical tests

For the catalase test, a loop full of the pure colony was transferred to a clean test tube containing 2 ml of 3% v/v hydrogen peroxide. According to Cheesbrough [18], the release of gas through the production of bubbles indicates

catalase positive while no reaction indicates catalase negative.

The coagulase test was carried out using the slide method as described by Cheesbrough [18]. At one end of the slide, mix a loop full of isolate with human plasma. For the control, mix a loop full of the same isolate with water at the other end of the same slide. The formation of aggregation after 30 sec will serve as confirmation for coagulase reaction.

The sugar fermentation test was done as described by Fngold and Baron [19]. The peptone water was prepared in a conical flask and the indicator bromocresol purple added to it. The contents were dispensed into a test tube containing Durham tubes and were sterilized at 121°C for 15 min. sugar solution (1%) was prepared and sterilized at 115°C for 10 min and then dispensed aseptically in 5 ml aliquot volume into the test tubes containing peptone water and indicator. The tubes were inoculated with the young culture of the isolate and incubated at 37°C for 24 h except for the control tube.

Indole test as Cheesbrough [20], described was done by inoculating the young culture of the isolates into test tubes containing 1 ml of peptone water and incubated for 48 h at 37°C, after which 4 drops of Kovac reagent was added to the test tubes and observed for colour change.

Haemolytic test as described in Chessbrough [18], was achieved by inoculating isolates in blood agar prepared with 5% sheep blood and incubated for 24 h at 37°C. The zone of haemolysis is checked and measured after the incubation period.

Oxidase test was done to identify if the isolate can produce cytochrome oxidase by oxidizing phenylenediamine. Glass rod was used to soak a piece of filter paper with few drops of oxidase reagent. Using a wire loop an inoculum was picked from the isolate and smeared on the soaked filter paper. Colour change was observed after 60 sec.

## 2.5 Antimicrobial Assay

### 2.5.1 Primary antimicrobial screening

Primary screening of the plant extracts for antibacterial activity was carried out using the

agar well diffusion method described by Biruhalem et al. [21], with little modifications. Dilutions of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/mL in DMSO (100% v/v) were prepared for each of the plant extract in a 2-fold dilution process. Mueller Hinton agar was prepared overnight and while isolates were reactivated and sub-cultured into blood agar. Isolates were picked from the overnight blood agar and introduced to a test tube containing 10 ml sterile normal saline and standardized by comparing to the 0.5 McFarland turbidity solutions. Using a sterile swab stick spread the aliquot aseptically on the overnight Mueller Hinton agar plates then allow the plate to dry under room temperature for about 30 minutes. Using a sterile cork-borer (6 mm), bore wells, 2 mm from the edge of the plate, and introduce the dilution of the extracts into the appropriate well and leave to diffuse into the agar for about 1 h then incubate at 37°C for 24 h. For antibacterial evaluation, Gentamicin (10 µg/ml) was used as positive control while DMSO (100% v/v) was used as the negative control. 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.

### 2.5.2 Analytical HPLC of the fractions

This was carried out according to the method of Ajaegbu et al., [22]. This was carried out with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany) at different lambda max (235, 254, 280 and 340 nm). Each sample (fraction) was dissolved using 2 ml of HPLC grade methanol, and 100 µl of the dissolved samples were each transferred into the vials of HPLC. 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 Nano pure water (adjusted to pH 2 by addition of formic acid) using methanol as the eluent. The absorption peaks for the fractions were analyzed by comparing it with those in the HPLC-UV/Visible library.

## 2.6 Statistical Analysis

The results were subjected to one way analysis of variance (ANOVA) followed by Turkey post

hoc multiple comparison test using SPSS software package version 23.0 to determine the significant differences. The results were expressed as mean  $\pm$ SD and statistically significant at P value < 0.05.

### 3. RESULTS AND DISCUSSION

#### 3.1 Extraction

Previous preliminary phytochemical screening indicates that *M. aboensis* is a rich source of flavonoids, phenolic acids, tannins, saponins, alkaloids, and steroids [23]. The percentage yield of water extract was greater than the percentage yield of ethanol extract in a report presented by Adonu et al., [24] on the phytochemical screening of the ethanol and water extracts of *M. aboensis*, *Cuscuta reflexa*, *Daniella oliveri*, and *Synclisia scabrida*. The methanol extraction from 90 g of powdered *Millentis aboensis*'s stem gave a yield of 2.94% w/w. The highest yield for the fractions was obtained from water (57.73% w/w) followed by ethyl acetate (16.79% w/w) and butanol (12.17% w/w) while hexane fraction gave the lowest yield (9.48% w/w) as shown in Table 1 below. The yields for the fractions were calculated from 18.5 g of methanol stem extract.

#### 3.2 Bacterial Isolate

*S. mutans* are predominant in patients under the age group of 18 – 25 years, and this may be as a result of frequent consumption of sugary beverages and food without restrictions [25]. From the 36 samples collected, *S. mutans* were present in 32 samples, 10 from male and 22 from females. This fact indicates that females are more prone to *S. mutans* than males. This can be attributed to the difference in the eating habits of male and female. The consumption of candy cake chocolate, gum, soft drinks ice cream is higher in females than in males [26].

Microscopic examination has shown that *S. mutans* is gram positive, cocci occurring in pairs or short- or medium length chains, with capsules. Also, they ferment glucose and lactose; it gives a negative result to catalase, oxidase, simmone citrate tests, motility, and urease, positive result to Vokes Proskeur and methyl red test [27]. The morphological examination indicates a circular raised cream colony that tests positive to gram stain,

appears as short rods in shape, and is immotile. The test isolate tested negative to catalase, oxidase, and indole test. It is coagulase positive and can ferment mannitol, glucose, and lactose.

#### 3.3 Antimicrobial Screening

The report was given by Onyegeme-Okerenta and Okafor [5], indicates no antimicrobial effect of *M. aboensis* extract against *E. coli*, but showed positive antimicrobial effects on *S. aureus*, *P. aeruginosa* and *K. pneumoniae* at 12.5 mg/ml – 400 mg/ml concentrations. The methanol extract and the fractions of *M. abonesis* stem did not show any sign of inhibition against *S. mutans* on the culture plates after 24 h while the conventional antibiotic showed significant inhibition zone against *S. mutans* even at the lowest concentration of 10  $\mu$ g/ml. The IZDs of the extract/fractions of the stem of *M. aboensis* was at 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/mL are against *S. mutans* are shown in Table 2.

#### 3.4 HPLC-DAD Analysis

Phytochemical analysis using HPLC-DAD analysis detected various constituents in the fractions of the methanol extract of the stem of *M. aboensis*. The HPLC screening revealed the presence of pestalotioprolide C - 1 and corynesidone D - 2 in MSWF (Fig. 1); enniatin B - 3 in MSHF (Fig. 2); dipiperamide E - 4, isopranetin 8-C-glucoside - 5, genistein 8-C-glucoside - 6 and genistein 6-C-glucoside – 7 in MSEF (Fig. 3); and peniciaculin B - 8 in MSBF (Fig. 4). Not much is known about pestalotioprolide C but corynesidone D can inhibit the production of NO and TNF- $\alpha$  and at the same time scavenging for reactive oxygen and nitrogen species [28]. Enniatin B is a secondary compound with ionphoric characteristics; it is produced by the fungi *Fusarium* and can serve as herbicidal, antifungal, insecticidal, and antibacterial compound. Following the report by Tsukamoto et al., [29] dipiperamides E has an inhibitory effect on drug metabolizing enzyme cytochrome P450 (CYP) 3AA while Isoprunetin can serve as an antioxidant [30,31]. According to Prosperini et al., [32] Antosiak et al. [33]. reported that Genistein-8-C-glucoside can suppress ovarian cancer cells due to its ability to inhibit the spread of cancer cells, induce apoptosis and generate reactive oxygen species.

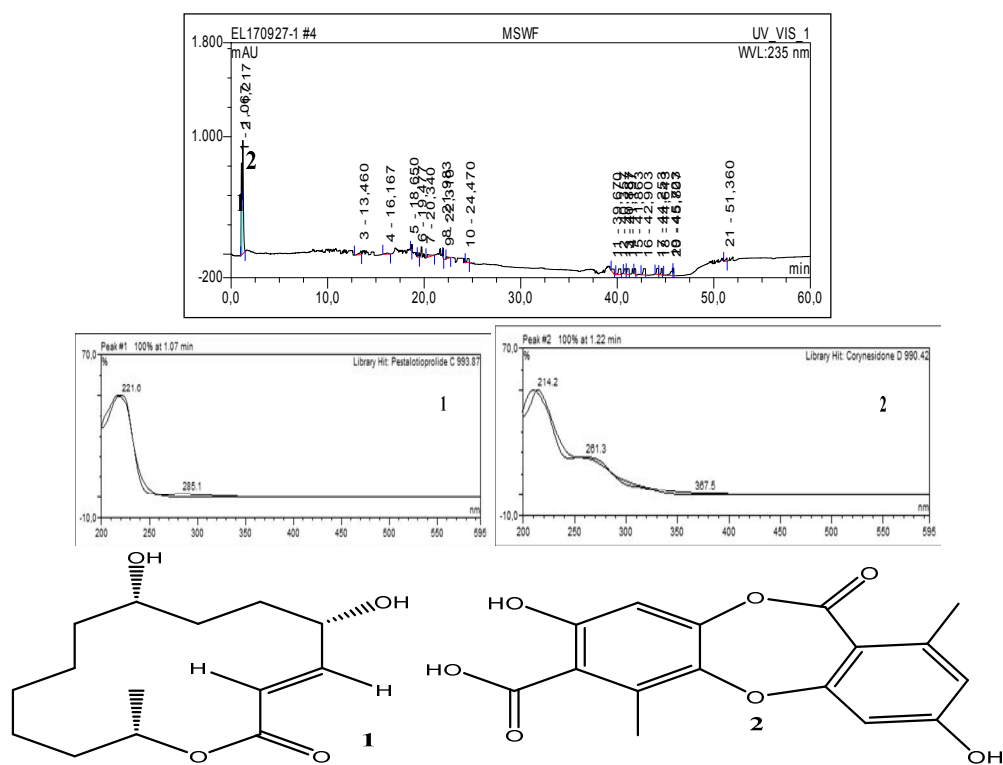


Fig. 1. HPLC chromatogram of MSWF showing pestalotioprolide C and corynesidone D; their UV spectra; and structures

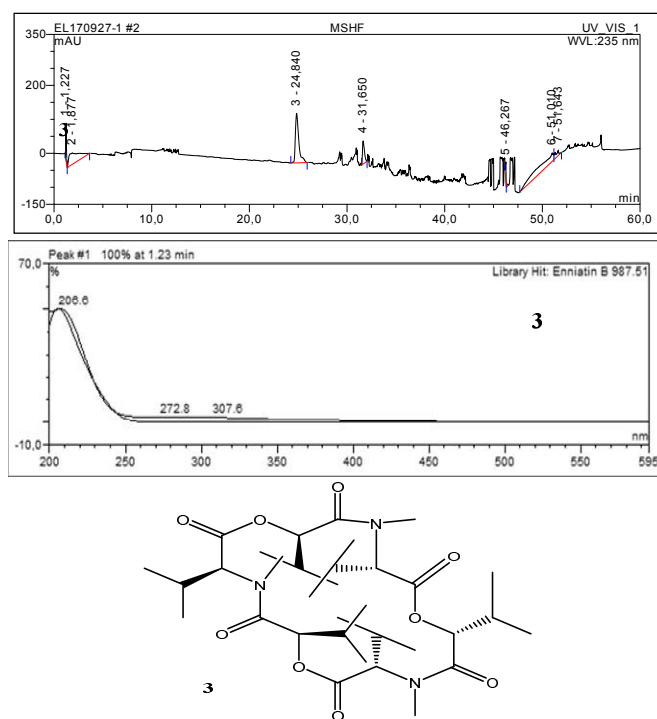
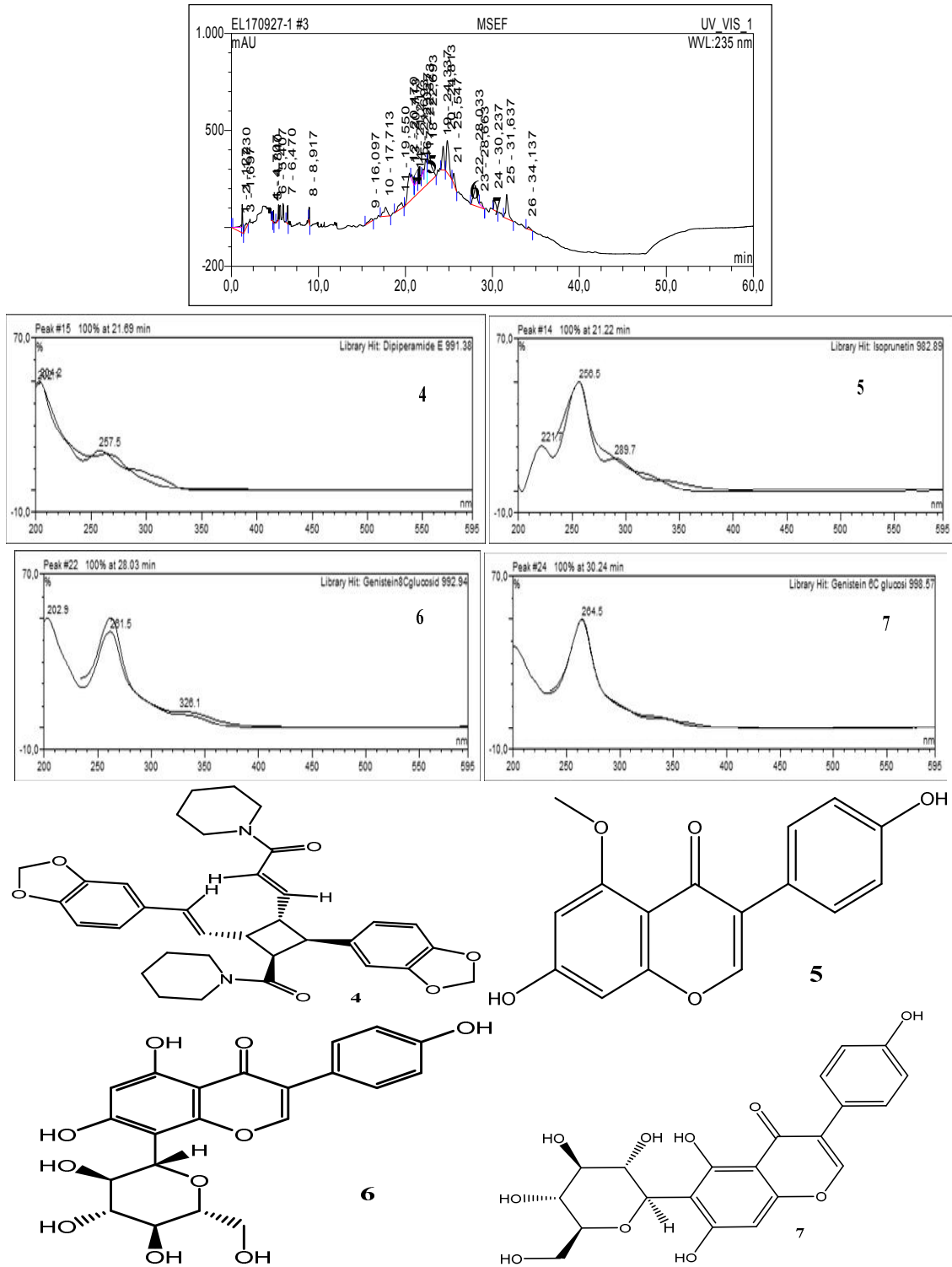


Fig. 2. HPLC chromatogram of MSHF showing enniatin B; its UV spectrum; and structure



**Fig. 3.** HPLC chromatogram of MSEF showing dipiperamide E, isopranetin 8-C-glucoside, genistein 8-C-glucoside, and genistein 6-C-glucoside; their UV spectra; and structures

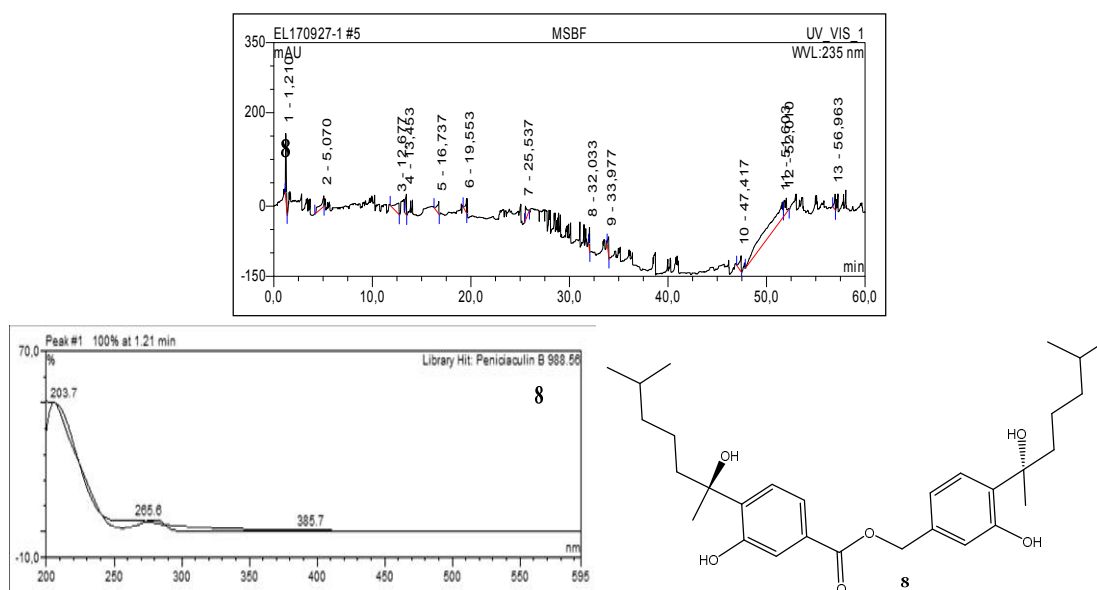


Fig. 4. HPLC chromatogram of MSBF showing peniciculin B; its UV spectrum; and structure

Table 1. Extract, Fraction and yields of extraction process of *Millettia aboensis* stem

Extracts	Yield (% w/w)
MSE <sup>a</sup>	2.94
MSHF <sup>b</sup>	9.48
MSEF <sup>b</sup>	16.79
MSBF <sup>b</sup>	12.17
MSWF <sup>b</sup>	57.73

MSE = Methanol stem extract, MSHF = Methanol stem hexane fraction, MSEF = Methanol stem ethyl acetate fraction, BSF = Methanol stem butanol fraction, MSWF = Methanol stem aqueous fraction. a = yield calculated from 90 g of plant material. b = yield calculated from 18.5 g of methanol stem extract

Table 2. Zone of inhibition in mg/ml produced by the crude extract of *Millettia aboensis* stem

Sample	Concentration (mg/ml)			
	6.25	12.5	25	50
MSE	0	0	0	0
MSHF	0	0	0	0
MSEF	0	0	0	0
MSBF	0	0	0	0
MSWF	0	0	0	0
Positive control gentamicin (10 µg ml)	-	-	-	25
Negative control DMSO	0	0	0	0

MSE = Methanol stem extract, MSHF = Methanol stem hexane fraction, MSEF = Methanol stem ethyl acetate fraction, MSBF = Methanol stem butanol fraction, MSWF = Methanol stem aqueous fraction.

#### 4. CONCLUSION

The stem of *M. aboensis* produces compounds with different biological properties from different fractions. Although the plant is said to have multipurpose medicine and can serve as an antimicrobial to some pathogenic microorganism, it does not affect *S. mutants* isolated from

patients with dental caries. Its compounds can be extremely useful in pharmaceutical industries as an active ingredient in the production of antibiotics.

#### CONSENT

It is not applicable.



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## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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