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Fagaramide and Pellitorine from the Stem Bark of Zanthoxylum zanthoxyloides and Their **Antimicrobial Activities**

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Authors' contributions

This work was carried out in collaboration among the authors. All authors read and approve the final manuscript.

Article Information

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ABSTRACT

Aim: Zanthoxylum zanthoxyloides is a plant of the family Rutaceae used for treating different ailments such as malaria, sickle cell anaemia, tuberculosis, paralysis and intestinal disorder due to the presence of some bioactive constituents. The present study was aimed at identifying and characterizing some of the active principles from the stem bark of the plant.

Places and Duration of Study: The isolation and characterization of the compounds was carried out at the Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathclyde, Glasgow, United Kingdom between October, 2018 to February, 2019 while the bioassay analysis was done at Ahmadu Bello University, Zaria, Nigeria.

Methodology: The stem bark powder was subjected to Soxhlet extraction with hexane to obtain the crude extract, which was fractionated on column using hexane, and ethyl acetate in increasing ratios. The isolated components were tested for their antimicrobial activities against some plants and animal pathogens at Ahmadu Bello University, Zaria, Nigeria.

Results: White crystals were obtained which on spectra analysis (IR, 1H-NMR, 13C-NMR, 2D-NMR) were identified as Fagaramide and Pellitorine The isolated compounds exhibited appreciable

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antimicrobial activities against some microbes, thus confirming the many ethnomedical uses of the plant.

Conclusion: The compounds isolated were identified as fagaramide and pellitorine. They showed moderate sensitivity towards the pathogens tested in the study.

Keywords: Zanthoxylum zanthoxyloides; fagaramide; pellitorine; zone of inhibition; MIC; MBC; MFC and pathogen.

1. INTRODUCTION

A great number of modern drugs have their origin from natural medicinal agents making traditional medicine an important tool that are potentially useful components for the development of chemotherapeutics [1]. However, the future of modern medicine and therapeutics could be linked to primitive medicinal practices as majority of new drugs have been inspired by natural products as well as compounds derived from natural products. Plants contain several chemical components including lipids, flavours, pigments, metabolites and so on which account for their uses as medicines and also as precursors for the development of modern drugs [2].

Zanthoxylum Zanthoxyloides is a shrub, spiny and more or less scandent, up to 6-8 m tall, with straight, often short bole and rounded and quiet dense crown; bark grey to beige, rough, with fine vertical fissures, often with woody pricklebearing protuberances. It is of the family Rutaceae in the tribe Zanthoxyleae containing two taxa namely; Fagara L and Zanthoxylum. The major characteristics of the Zanthoxylum family is that the trunks, branches, branchets, leaf stalks and inflorescence axes of all these species are covered by prickles or what is also called spines [3]. The plant is popularly known as Faschuari in Hausa, Rapeko in Moore, Gozo Ngua in Bambara, Aminpee in Ogoni. The plant considered analgesic, antiseptic diaphoretic [4]. The stem bark aqueous extracts are widely used for the treatment of malaria, fever, sickle cell anaemia, tuberculosis, paralysis, oedema and general body weakness Cameroon [4]. The root is used in the treatment of intestinal problems including colic, dysentery, intestinal worms, gonorrhoea, urethritis as well as stimulants. In southern Nigeria, a decoction of the stem bark and roots is used to treat cancer, swellings, wounds, leprosy and syphilitic sores as well as rheumatic and arthritic pain and hernia [5]. The fruits of the plant have been reported to contain α -pinene trans- - β -ocimene, citronellol, geraniol, limonene and β-myrcene [6]. The present study investigated the stem bark of the

plant for its chemical contents that could be responsible for the reported medicinal potencies of the plant as well as its antimicrobial activity against some clinical and plants pathogens.

2. MATERIALS AND METHODS

The bark of Zanthoxylum zanthoxyloides was collected from Bunu Tai, Tai LGA, in Rivers state. The plant material was identified at the Department of Forestry and Environmental studies, Rivers State University, Port Harcourt. The sample was assigned voucher specimen number RSU/2017/ZZ-56 and deposited in their herbarium. The stem bark was air dried and ground to powder using mortar and pistol.

2.1 Extraction

The pulverised Zanthoxylum Zanthoxyloides stem bark (1 kg) was extracted by soxhlet apparatus using hexane for 72 hours. The extract was evaporated to dryness in rotary evaporated at 40°C.

2.2 Isolation and Characterisation

The crude hexane extract (10 g) was dissolved in hexane (30 mL) and absorbed on to silica gel (8 g) then the solvent was allowed to evaporate completely to form a slury. The slury was applied as a concentrated band onto a gravity column and eluted gradient wise, starting with hexane (200 mL) followed by mixtures of hexane; ethyl acetate 90:10, 85:15, 80:20 (200 mL each) [7]. Fractions were collected in 20 mL vials and allowed to stand until the solvents evaporated to dryness at room temperature. Similar column fractions were combined after TLC analysis [8]. Fractions 29,30 and 31 eluted with hexane; ethyl acetate gave similar TLC profile single greenish spots when charred with concentrated sulphuric acid, RF values 0.63. Similarly, fractions 11,12,13 and 14 eluted with hexane; ethyl acetate gave similar TLC profile (brown spots when charred), RF values 0.61. The combined fractions 29-31 and 11-14 were recrystallized in ethyl acetate to yield compounds 1 and 2 labelled PJZS-29 and PJZS-13 respectively. The compounds were subjected to spectroscopic analysis (NMR Spectroscopy and mass spectrometry), their melting points were also determined as well as bioassay to ascertain their biological activities against some pathogens.

2.3 Antimicrobial Screening

The antimicrobial activities of the isolated compounds from the plant under investigation were determined using some animal and plant pathogens. The animal pathogens were obtained from the Department of Medical Microbiology Ahmadu Bello University teaching hospital, Zaria. The plant pathogens were obtained from I.A.R A.B.U. Zaria. The animal pathogens (bacteria) used for the antimicrobial assay include; Methicillin Resist staph aureus, Vancomycin resist enterococci. Staphylococcus aureus. Escherichia Salmonella coli. tryphi, Pseudomonas aeruginosa. The plant pathogens (fungi) used in this study were, Aspergillus flavus, Aspergillus fumigatus, Aspargillus nigre, puteana, Coniophora Fibrophoria vaillentii, **Fomitopis** pinicola, Fusarium oxysporum, Fusaruim proliforatum, Rhizopus sp., Sclerotium rofsii and Serpula lacrymans. All microorganisms were checked for purity and maintained in slants for agar [9].

2.4 Cultivation and Standardization of Test Organism

A loop full of test organisms were taken from the agar slant and sub culture into test tubes containing sterile nutrient agar for bacterian and subourand dextrose medium (20 ml) for for fungi, the test tubes were incubated for 48 hrs at 37°C. The broth cultures were standardized using sterile normal saline to obtain a density of 106 cfu/mL for bacteria. A sporulated test fungal spore was harvested with 0.05% Tween 80 in sterile normal and standardized to 106 spores/mL.

2.5 Antimicrobial Profile (Sensitivity Test)

0.002~mg of the compound was weighed and dissolved in 10 mls of DMSO obtain a concentration of 2 µg/ml. Diffusion method was used for screening the compound. Mueller Hinton agar sabouraud dextrose agar were the media used as the growth media for the microbes. The media were prepared according to the manufacturer's instructions sterilized at 121°C for

15 minutes, poured into sterile petri dishes and were allowed to cool and solidify. The Mueller Hinton agar was seeded with 0.1 ml of the standard inoculum of the test bacteria while the sabouraud dextrose agar was seeded with 0.1 ml of the test fungi. The inoculum was spread evenly over the surface of the medium by the use of a sterile swab. By the use of a standard sterile cork borer of 6mm in diameters, well was cut at the centre of each inoculated media. 0.1 ml of solution of the compound concentration of 20 µg/ml was then introduced into the well on the inoculated media. Incubation was made at 37°C for 24 hrs for the bacteria and at 30°C for 1-7 days for the fungi after which the plates of the media were observed for the zone of inhibition of growth, the zone was measured with a transparent ruler and the result recorded in millimetres [10].

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibition concentration of the compounds was determined using the broth dilution method. Mueller Hinton broth and sabouraud dextrose broth were prepared, 10 mL of the broth was dispensed into test tubes and were sterilized at 121°C for 15 minutes, the broth was allowed to cool. Mc-farland's turbidity standard scale number 0.5 was prepared to give turbid solution. Normal saline was prepared, 10 mL was dispensed into sterile test tube and the test microbe was inoculated and incubated at 37°C for 6hrs. Dilution of the test microbe was done in normal saline until. The turbidity marched that of Mc-farland's scale by visual comparison at this point the test microbe has a concentration of 1.5 ×10⁸ cfu/ml. Two-fold seriel dilution of the compound was done in the sterile broth to obtain the concentrations of 20 µg/m/l, 10 µg/ml, 5 μg/ml, 2.5 μg/ml and 1.25 μg/ml. the initial concentration was obtained by dissolving 0.002 mg of the compound in 10 mls of the sterile broth. Having obtained the different concentrations of the compound in the sterile broth, 0.1 ml of the test microbe in the normal saline was then inoculated into the different concentrations incubation was made at 37°C for 24 hours for the bacteria and at 30 °C for 1-7 days for the fungi, after which the test tubes of the broth were observed for turbidity (growth) the lowest concentration of the compound in the sterile broth which shows no turbidity was recorded as the minimum inhibition concentration (MIC) [11].

2.7 Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

MBC/MFC was carried out to determine whether the test microbes were killed or only their growth inhibition. Mueller agar and sabouraud dextrose agar were prepared, sterilized at 121°C for 15 minutes, poured into sterile petri dishes and were allowed to cool and solidify. The contents of the MIC in the seriel dilutions were then sub-cultured onto the prepared media, the bacteria were subcultured onto the Mueller agar while the fungi were sub-cultured onto sabouraud dextrose agar, incubation was made at 37°C for 24 hours for the bacteria and at 30°C for 1-7 days for the fungi, after which the plates of the media were observed for colony growth MBC/MFC were the plates with lowest concentration of the compound without colony growth. Results were recorded after 24 hours [9].

3. RESULTS AND DISCUSSION

3.1 Characterization of PJZS 13 as Fagaramide

Fraction PJZS- 13 was obtained as cream crystals with a melting point of 113-115°C. It absorbed UV when visualized after TLC in a UV-lamp. The $^1\text{H}-\text{NMR}$ spectrum showed a spin system for two olefinic protons at δ_H 6.3 ppm (H-7) and δ_H 7.35ppm (H-8). The large coupling

constant (J = 15.6Hz) exhibited between H-7 and H-8 was consistent with trans configuration of the olefinic bond. Furthermore, the $^1\text{H-NMR}$ displayed signals at δH 3.32 ppm (-CH $_2\text{-m}$), 1.87 ppm ($^1\text{H,m}$), 0.93 (CH $_3$) $_2$, (d, J = 7.2Hz) indicating the presence of an isobutylamide chain in the compound. The spectrum also showed the presence of three mutually coupled aromatic protons at δ_H 6.71 (H-2) and δ_H 6.93 (dd J = 8.2, 2.0 Hz, H-6) and δ_H 6.91ppm (d, J = 8.2 Hz, H-5) of a tri-substituted benzene ring.

The $^{13}\text{C-NMR}$ spectrum confirmed the presence of 14 carbon peaks. These peaks revealed the presence of six aromatic carbons of which three (3) were CH appearing at δc 120.5 (C-2), δc 123.6 (C-5) and δc 129.5 (C-6) ppm respectively. Three (3) quaternary carbons at $\delta_{\rm C}138.7$, δc 147.3 and δc 148.1 ppm assigned to C-1, C-3 and C-4 respectively. There was also a signal with $\delta_{\rm C}101.6$ ppm corresponding to the methylene-dioxy group, a signal at $\delta_{\rm C}166.14$ ppm for the olefinic carbons, lowfield CH₂ at $\delta_{\rm C}$ 46.5 ppm near to the nitrogen atom. Signals for methyl groups were observed at δc 20.3 and $\delta_{\rm C}$ 21.3 ppm assigned to C-3' and C-4' respectively were also observed [12].

Using the above ¹H and ¹³C-NMR spectral data and comparison with literature reports, fraction PJZS 13 was characterized and identified as fagaramide. This compound has been isolated previously from a number of plants including *Z. schinifolium* [12].

Table 1. ¹HNMR and ¹³C NMR chemical shifts for PJZS-13

Spectroscopic technique	Data
Rf	0.61
Mp ¹ HNMR(Acetone)	113-115°C
¹ HNMR(Acetone)	δ 6.71(H-2), 6.91(H-5), 6.93(H-6), 6.3(H-7), 7.35(H-8), 3.32(H-1 ¹),
	$1.87(H-2^{1}), 0.98(H-3^{1}), 0.85(H-4^{1}), 6.04(OCH_{2}O), 3.94(NH)$
¹³ C NMR(Acetone)	δ 138.7(C-1), 120.5(C-2), 147.3(C-3), 148.1(C-4), 129.5(C-5),
	123.6(C-6), 106.3(C-7), 108.8(C-8), 166.4(C-9), 46.5(C-1 ¹), 28.4(C-2 ¹),
	20.3(C-3 ¹), 21.3(C-4 ¹), 101.6(OCH ₂ O)

Fig. 1. Chemical structure of compound PJZS-13 (Fagaramide)

3.2 Characterisation of PJZZ 80-85 as Pellitorine

The fraction coded PJZZ 80-85 was isolated as a white crystals and with molecular formula C₁₄H₂₅NO gave a molecular ion peak at M/z 223.0 in the E1-Ms spectrum. The infrared spectrum showed a strong absorption band at 3300cm⁻¹, which accounted for the presence of the NH group, a strong absorption band was observed at 1656cm⁻¹, belonging to the C=0 of the amide group. Its melting point was obtained at 61-63°C. The ¹H-NMR spectrum of PJZZ 80-85 showed three doublet signals at $\delta_H 5.78$ ppm for H-2, δ_H 0.89 for H-3¹, and δ_H 0.84ppm for H- 4^{1} . Two multiplet signals, which appeared at δ_{H} 6.05ppm and $\delta_H 6.01$ ppm, were due to the olefinic protons at C-4 and C-5. However, a multiplet occurring δ_H 1.79ppm, was due to the proton at C-2', coupling with the adjacent protons in the isobutyl moiety. A broad singlet at δ_{H} 5.74ppm was attributed to NH.

The $^{13}\text{C-NMR}$ spectrum gave a total of 14 peaks and most of the carbon peaks appeared at the upfield region. The amide carbonyl carbon resonating at $\delta_{\text{C}}166.60$ ppm was assigned C-1. A ^3J correlation between δ_{C} 166.60 (C-1) and δ_{H} 3.13ppm (H-1 1) was observed and it validated

the position of the isobutyl group. The location of the olefinic protons was confirmed by a 3J -correlation to the carbonyl carbon at $\delta_{\rm C}$ 166.60 ppm from a proton at $\delta_{\rm H}$ 5.78 ppm. The chemical shifts of the proton and carbon NMR spectra were compared with literature values, and the values for both 1H and $^{13}C\text{-NMR}$ spectra were found be in agreement. Thus, fraction PJZZ 80-85 was identified as deca-2E, 4E-dienoic acid isobutyl amide (Pellitorine).

Form the biological studies, Aspergillus flavus, Aspergillus fumigates, Coniophora puteana, Fibrophoria vaillentis, Fusarium proliferation and Rhizopus Sp were sensitive to the compound PJZZ 80-85 (Table 3) while Aspergillus nigre. Fomitropsis Pinicola, Furisarium oxysporum, Sclerotium rofsii and Serpula lacrymas were resistant to compound PJZZ 80-85. The result showed that the organisms (fungi) exhibited and inhibition zone between 26-29 mm which was consistent with the vended drugs ranging from 26-32mm for fulcin and 23-28 mm ketoconazole. Coniophora puteana, Fibrophoria vailentii, Serpula lacrymans was sensitive to PJZS-13 whereas the rest organisms under investigation were resistant to the compound. The zone of inhibition ranges from 30-32 mm (Table 3).

Table 2. ¹HNMR and ¹³C NMR chemical shifts for PJZZ-80-85

Spectroscopic technique	Data
1R(cm ⁻¹)	3300, 1656
Rf	0.58
Мр	60 – 62°C
EI – Ms	223.0
¹ HNMR(Acetone)	δ 5.78(H-2), 7.16(H-3), 6.05(H-4), 6.10(H-5), 2.14(H-6), 1.39(H-7),
	$1.26(H-8)$, $0.89(H-10(, 3.13(H-1^1), 1.79(H-2^1), 0.89(H-3^1), 0.84(H-4^1),$
	5.74(NH)
¹³ C NMR(Acetone)	δ 166.60 (C-1), 121.84(C-2), 141.40(C-3), 128.30(C-4), 128.30(C-
	5),33.02(C-6), 28.71(C-7), 31.46(C-8), 22.58(C-9), 14.13(C-10),
	47.02(C-1 ¹), 28.57(C-2 ¹), 20.24(C-3 ¹), 20.24(C-4 ¹)

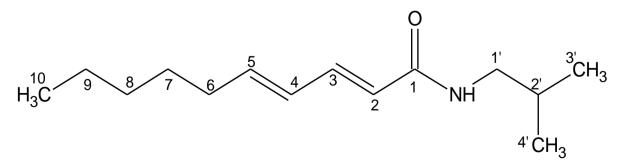


Fig. 2. Chemical structure of compound PJZZ: 80-85 (Pellitorine)

Table 3. Antifungal and sensitivity test, zone of inhibition (mm) of PJZZ80-85 and PJZS-13 versus vended drugs

Text organisms	PJZZ 80-85	PJZS 13	Fulcin	Kefeconazole
Aspergillus flavus	S (27)	R (0)	S (26)	R(0)
Aspergillus fumigates	S (29)	R (0)	S (28)	R (0)
Aspargillus nigre	R (0)	R (0)	S (30)	S (25)
Coniophora puteana	S (26)	S (30)	R (0)	S (23)
Fibrophoria vaillentii	S (28)	S (32)	S (28)	R (0)
Fomitopsis pinicola	R (0)	R (0)	S (30)	S (27)
Fusarium oxysporum	R (0)	S (31)	S (32)	R (0)
Fusarium proliforatum	S (29)	R (0)	S (27)	R (0)
Rhizopus SP	S (27)	R (0)	S (26)	S (28)
Sclerotium rofsii	R (0)	S (30)	R (0)	S (25)
Serpula lacrymans	R (0)	S (30)	S (30)	S (26)

Table 4. Antibacterial and sensitivity test, zone of inhibition (mm) of PJZZ 80-85 and PJZS 13 versus vende drugs

Text organism	PJZZ 80-85	PJZS 13	Sporfloxacin	Ciprofloxaxin
Methicillin resist staph aureus	S (26)	R (0)	S (30)	R (0)
Vancomycin resist enterococci	S (28)	S (29)	S (29)	S (30)
Staphylococcus aureus	R (0)	S (30)	S (32)	S (26)
Escherichia coli	R (0)	R (0)	S (27)	S (37)
Salmonella typhi	S (28)	S (31)	R (0)	S (40)
Pseudomona aeruginosa	S (30)	S (30)	R (0)	S (25)

Key: R = Resistance; S = Sensitive

Table 5. Minimum inhibitory concentration of PJZZ 80-85 and PJZS 13

Text organisms	PJZZ 80-85	PJZS 13	
	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.25 mg/ml	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.25 mg/ml	
Aspergillus flavus		0* +	
Aspergillus fumigates			
Aspargillus nigre			
Coniophora puteana	0* +	0* +	
Fibrophoria vaillentii	0* +		
Fomitopsis pinicola			
Fusarium oxysporum	0* +	0* +	
Fusarium proliforatum		0* +	
Rhizopus SP		0* +	
Sclerotium rofsii	0* +		
Serpula lacrymans	0* +		

Key: -= No turbidity (No growth), 0 = MIC, += turbid (light growth), += moderate growth

The antibacterial result showed that Methicillin resists staph aureus, Vancomycin resist enterococci, Salmonella typhi and Pseudomona aeruginosa exhibited high sensitivity towards PJZZ 80-85 with inhibition zones ranging from 26-30 mm whereas Staphylococcus aureus and Escherichia coli were resistant. However, Methicillin resist staph aureus and Escherichia

coli were resistant to PJZS13 while other microorganisms used were sensitive to PJZS-13. The results obtained were in consistent with that obtained when compared with the controlled drugs. The organisms exhibited an inhibitory zone ranging from 26-30 mm against PJZZ80-85 and 29-31 mm against PJZS13 (Table 4). Howbeit, Salmonella typhi exhibited the highest

zone of inhibition against PJZS13 while the highest zone of inhibition against PJZZ 80-85 was noticeable by *Pseudomona aeruginosa*. The

sensitivity potencies of the microorganisms are evidences for numerous traditional medical uses of the plants.

Table 6. Minimum fungicidal concentration of PJZZ 80-85 and PJZS 13

ext organisms PJZZ 80-85		PJZS 13	
	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.5 mg/ml	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.5 mg/ml	
Aspergillus flavus		0* + ++	
Aspergillus fumigates			
Aspargillus nigre			
Coniophora puteana	0* ++ +++	0* + +++	
Fibrophoria vaillentii	0* ++ +++		
Fomitopsis pinicola	0* ++ +++	0* + ++	
Fusarium oxysporum	0* + ++		
Fusarium proliforatum		0* + ++	
Rhizopus SP		0* + ++	
Sclerotium rofsii			
Serpula lacrymans	0* + ++		

Key: -= No colony growth, 0 = MBC, += scanty colonies growth, ++= moderate colonies growth, +++ = heavy colonies growth

Table 7. Minimum inhibitory concentration of PJZZ 80-85 and PJZS 13

Text organisms	PJZZ 80-85	PJZS 13
	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.25 mg/ml	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.25 mg/ml
Methicillin resist staph aureus		0* +
Vancomycin resist enterococci	0* +	
Staphylococcus aureus	0* +	0* +
Escherichia coli		0* +
Salmonella typhic	0* +	
Pseudomona aeruginosa	0* +	0* +

Key: -= No turbidity (No growth); $0^* = MIC$; += Turbid (light growth); ++= Moderate turbidity

Table 8. Minimum bactericidal concentration (MBC) of PJZZ 80-85 and PJZS 13 against test organism

Text organisms	PJZZ 80-85	PJZS 13
	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.25 mg/ml	20 mg/ml 10 mg/ml 5 mg/ml 2.5 mg/ml 1.25 mg/ml
Methicillin resist staph aureus		0* ++ +
Vancomycin resist enterococci	0* ++ +	
Staphylococcus aureus	0* ++ +	0* ++ +
Escherichia coli		0* ++ +
Salmonella typhic	0* ++ +	
Pseudomona aeruginosa	0* ++ +	0* ++ +

Key: - No colory growth $0^* = MBC + = scanty$ colonies growth, ++ = moderate colonies growth

4. CONCLUSION

This present study investigated the isolation and characterization of Fagaramide and pellitorin from Zanthoxylum zanthoxyloides stem bark using physical and spectroscopic methods. The compounds were tested against clinical and plant pathogens and the results an appreciable antibacterial antifungal activities, thus, affirming the tradomedical uses of the plant. This is an evidence for the use of the isolated compounds as drug potential candidates for treatment of several fungi and bacteria diseases caused by the pathogens tested in the study. However, the study also revealed that the compounds could also serve as potential candidates for pesticides production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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