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Original Research

Antioxidant, antibacterial and antibiofilm activities of ethanol extract of *Zapotecca portoricensis* roots

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ABSTRACT

This study evaluated the phytochemical composition, antioxidant, antibacterial and antibiofilm activities of ethanol extract of *Zapotecca portoricensis* root. The extract was prepared via cold maceration method and gas chromatography-mass spectrometry (GC-MS) was used to determine the phytochemical composition of the extract. The antioxidant activity was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) procedures. The antibacterial and antibiofilm activities of the extract were evaluated against clinical isolates of *Eschericia coli*, *Staphylococcus aureus* and *Salmonella typhi* using agar well diffusion technique. The GC-MS analysis showed the presence of seven (7) bioactive compounds. The extract produced concentration dependent increase in the antioxidant values in both the DPPH and FRAP assay protocols. The extract produced concentration-dependent increase in the zone of inhibition against the tested organisms. At 62.5 mg/mL and below concentrations, the zones of inhibition of *Z. portoricensis* root extract against *E. coli* and *S. aureus* were significantly ($p < 0.05$) lower when compared with the zone of inhibition of *Z. portoricensis* root extract against *S. typhi*. The minimum inhibitory concentration (MIC) of *Z. portoricensis* root extract against *E. coil, S. aureus* and *S. typhi* were 31.25, 15.63 and 62.50 mg/mL concentration, respectively. The extract produced concentration-dependent increase in antibiofilm activity. At 7.5 mg/kg, the extract produced 28.87, 34.01 and 66.45% inhibition of biofilm production against *S. typhi*, *E. coli* and *S. aureus*, respectively. The extract possess antioxidant, antibacterial and antibiofilm activities which could be linked to the presence ethyl iso allocholate and hexadecanoic acid.

Keywords: antioxidant; antibacterial; antibiofilm; hexadecanoic acid; *Zapotecca portoricensis*

INTRODUCTION

The use of plants in the management and treatment of diseases is as old as human existence on earth (Manandhar *et al*., 2019). The biological activities of medicinal plants are influenced by their phytochemical composition (Prinsloo & Nogemane, 2018). These phytochemicals such as alkaloids, saponins, tannins, cardiac glycoside, flavonoids etc are influenced by geographical location, season, plant parts, time of collection and climatic factors (Adamczak *et al*., 2020). These phytochemicals are produced by the plant as protective agent against external stressor and pathogenic attacks, hence useful for plant's defense and survival (Jan *et al*., 2021). The World Health Organisation estimated that about 80% of the world population depend on traditional medicine or drugs of plant origin in attending to their healthcare needs (Hosseinzadeh *et al*., 2015).

Bacterial diseases are commonly treated with medicinal plants. The growing interest in traditional medicine is attributed to cost, unavailability and resistance of pathogenic bacteria to orthodox drug in clinical practice (Adedeji *et al*., 2023). Resistance is one of the challenges facing the combating of bacterial infections in clinical practice (Murray *et al*., 2021). One of the processes that enhance the resistance of bacteria to antimicrobial agent is the formation of biofilms. Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces (Sánchez *et al*., 2016). They are considered an important virulence factor that is accountable for persistent chronic and recurrent infections and are strongly resistant to antibiotics and host immune defenses (Hordofa, 2022). Biofilm resistance to standard treatments is increasing, necessitating the development of new control measures.

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Biofilm inhibition is a popular medication target for the cure of a variety of bacterial and fungal illnesses, and the pharmacological development of these therapies is now being researched extensively. Many green non-lethal biofilm management tactics have been established in recent years, making the mode of action of these novel antibiofilm agents far less prone to resistance (Sánchez *et al*., 2016). One interesting alternative is to look for naturally occurring plant-derived chemicals that can prevent biofilm formation.

Zapoteca portoricensis, commonly called white stick or "nnu-nde" or "elugelu" in Nsukka South-East Nigeria, belong to the family Fabaceae (Ikeyi *et al*., 2020). It is a perennial shrub with thin branches, small oval green leaves, cream colored flowers and flat fruits. It is mostly found in West Africa particularly in Togo (Misahohe), Southern Nigeria (Bonny, Oban, Aguku and Lagos) with Ghana (Odumase, Aburi), as well as West Indies and the Atlantic Coast of America (Agbafor *et al*., 2014). The leaves are used in the treatment of tonsillitis, spasmodic and other gastrointestinal disorders, while the root possesses anti-inflammatory, antifungal, and antibacterial activities (Agbafor *et al*., 2014). The decoction of *Z. portoricensis* is utilized in Eastern Nigeria in the traditional management of disorders like constipation, convulsion, prolonged labor, typhoid fever, abdominal discomfort, snake bite and as antipoison (Ikeyi *et al*., 2020). Despite the wide use of *Z. portoricensis* in the folk treatment of bacterial disease, there is limited classical information in scientific literature on its antibiofilm and antioxidant activities. This study aimed to investigate the phytochemical composition, antioxidant, antibacterial and antibiofilm activities of the ethanol extract of *Z. portoricensis* root against some selected pathogenic organisms.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIALS

The *Z. portoricensis* roots sample were collected from botanical garden of late Chief Thomas Eze in Olido Enugu-Ezike, Igbo-Eze North L.G.A of Enugu state. The sample was authenticated by Mr A. O. Ozioko of Bioresources Development and Conservation Programme, Nsukka, Enugu State.

PREPARATION OF THE PLANT EXTRACTS

The Z. *portoricensis* root extract (ZPRE) was prepared following the methods of Tarek *et al* (2019) with little modification. 10 g of air dried plant parts powder were soaked in 150 ml of 90% ethanol and allowed to stand for 48 hours. Thereafter, the extract was filtered through Whatmann No 1 filter paper and evaporated in hot air oven at 45 °C. Stock solutions were prepared in dimethyl sulphoxide (DMSO) and stored at 4°C in the refrigerator.

GAS CHROMATOGRAPH-MASS SPECTROMETRY (GC-MS) OF PLANT EXTRACTS

The ZPRE was analyzed by Gas Chromatography Mass (GC-MS) using a HP-5MS capillary column (30ml 250µm, i.d., 0.25 µm film thickness) in an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a water GCT Premier mass spectrometer (Waters Corporation, Milford, MA, USA). The carrier gas was helium with a constant flow rate of 3 mL/min. The oven temperature was initially kept at 100 °C for 4 min then ramped at 10 °C/min to 240 °C. The temperature was gradually increased from 8 °C/min to 300 °C and held isothermally for 10 min. 1.0 mL of the sample (100 ppm in chloroform) solutions was injected in the split-less mode. Mass spectra were obtained by electron ionization at 70 eV over the scan range of 50-800 m/z. The compounds were identified by comparison of their mass spectra with those of the NIST 05 L mass spectral library. The spectral match factor limit was set at 700 and any components with match factor less than 700 were not considered.

ANTIOXIDANT TEST

2, 2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) PHOTOMETRIC ASSAY PROCEDURE

The free radical scavenging activity of the extract was investigated by the DPPH assay (Mensor *et al*., 2001) using spectrophotometer. The crude extracts at concentrations (25, 50, 100, 200 and 400) µg/mL each was mixed with 1 mL of 0.5 mM DPPH (in methanol) in a cuvette. The absorbance at 517 nm was taken after 30 minutes of incubation in the dark at room temperature. The experiment was done in triplicate. The percentage antioxidant activities were calculated as follows.

% antioxidant activity (AA) = 100-[{(ABS sample—ABS blank) ×100}/ABS control]

One mililitre of methanol plus 2.0 mL of the test extract was used as the blank while 1.0 mL of the 0.5 mM DPPH solution plus 2.0 mL of methanol was used as the negative control. Ascorbic acid (vitamin C) was used as reference standard.

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) PROCEDURE

The ferric reducing antioxidant power was carried out as described by Benzie and Strain, (1999). FRAP working solution was prepared by mixing solution 1, 2 and 3 in the ratio of 10:1:1, respectively. The working solution was freshly prepared. The FRAP reagent (3 mL) and 100 µl sample solution at concentrations of 25, 50, 100, 200 and 400 µg/mL was mixed and allowed to stand for 4 minutes. The absorbance was recorded at 593 nm, at 37°C. The ascorbic acid was tested in a parallel process. The absorbance of each test tube was taken at 0 and 4 minutes after addition of sample.

FRAP value = $abs\ 4\,\text{minutes} - abs\ 0\ minute$

IDENTIFICATION AND CONFIRMATION OF THE TEST ORGANISMS

Clinical isolates of *Eschericia coli, Staphylococcus aureus* and *Salmonella typhi* used were obtained from Microbiology Laboratory in Federal Medical Center (FMC) Umuahia, Abia State and was maintained in brain heart infusion agar slant at 4°C and were confirmed using selective media, Gram stain and biochemical tests. The various isolates were cultured accordingly on the following: *E. coli* in Eosin methylene blue (EMB), *Salmonella typhi* in *Salmonella* Shigella agar, *Staphylococcus aureus* in Mannitol Salt agar and Muella Hilton agar for sensitivity test and broth for minimum inhibitory concentration (MIC). The isolates were inoculated on the culture media using streak plate method and were incubated at 37°C for 24 hours. Colony of the growth was transferred to a pure culture plate, and incubated at 37 °C for another 24 hours, biochemical identification was done using Cheesbrough (2005) method.

EVALUATION OF ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS

Antimicrobial activity of the plant extract was evaluated using agar well diffusion method. Fifty microlitres of fresh culture was uniformly spread onto Mueller Hinton agar plates by sterile Driglasky loop. The inoculated plates were allowed to dry at room temperature for 20 minutes, thereafter, wells of 6 mm in diameter were made using the sterile cook borer and 25 µl of each extracts was aseptically pipetted into the wells. Ciprofloxacin was used as control. Plates were incubated at 37°C for 24 hours. Antibacterial activity was recorded according to the zone of inhibition which was measured and recorded in millimeters (CLSI, 2021).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

A serial dilution of the plant extract was performed using sterile diluent of Mueller Hinton broth and 25 µl of the inoculums was added to each test tube. The inoculated tubes were incubated at 37 °C for 24 hours. 2,3,5-triphenyl tetrazolium chloride (TTC) was added to the tubes and the tubes were incubated one and half hours. TTC is known to be colorless and turns red when biologically active bacteria have grown. Inhibition was detected when the solution in the tube remain colorless with TTC.

BIOFILM INHIBITION ASSAY

The *S. aureus, E. coli* and *S. typhi* from fresh agar were inoculated in Brain Heart Infusion broth with 1% glucose and incubated for 24 hours at 37 °C in stationary state at concentrations below the MIC of the test isolates. 96 wells U bottom tissue culture plates were filled with each extract and 100 µl of suspended inoculums were added and incubated overnight at 37 °C, after which the well content was washed with sterile distilled water to remove planktonic cells. Adherent cells in plates were stained with 0.1% crystal violet for 10 minutes. Excess stain was rinsed off with distilled water and plates were air dried and resuspended in 100µl of 95% methanol. The optical density of the stained adherent cells was determined with ELISA microplate reader at 630 nm wavelength (Bazargani *et al.*, 2016). The percentage of inhibition was then compared with the control

$$\textbf{Inhibition \%} = \frac{\textbf{1−A630 of the test}}{\textbf{1− A630 of non−treated control}} \times \frac{\textbf{100}}{\textbf{1}}$$

DATA ANALYSES

Data were entered into excel sheet for consistency check and then exported to SPSS 20 for Windows (SPSS Inc., Chicago, USA) for analysis according to the objectives of the study. Analysis of variance (ANOVA) and statistical significant differences were determined using Duncan multiple range test. P-value < 0.05 was considered statistically significant.

RESULTS

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF ZPRE

The result of the GC-MS analysis of the ZPRE is presented Table I. Seven bioactive compounds; pentachlorobenzonitrile, ethyl iso allocholate, phorbol, hexadecanoic acid, 3-Pyridire carboxylic acid, Gibb-3-ene-1, 10 docarboxylic acid, and Tatradecanoic acid were identified.

Table I: Gas chromatography-Mass spectrometry analysis of ZPRE

S/N	RT (min)	Compound	MF	MW
1	4.49	Hexadecanoic acid (palmitic acid)	$C_{36}H_{58}O_6$	586
2	5.22	3-Pyridine Carboxylic acid	$C_{36}H_{58}O_6$	597
3	9.57	Gibb-3-ene-1,10 dicarboxylic acid	$C_{10}H_{24}O_6$	360
4	10.49	Tetradecanoic acid	$C_{31}H_{50}O_6$	518
5	14.57	Pentachloro benzonitrile	C_7Cl_5N	248
6	13.64	Ethyl iso allocholate	$C_{26}H_{44}O_5$	436
7	13.63	Phorbol	$C_{20}H_{28}O_6$	364

MF = Molecular formular, MW = Molecular weight, RT = Retention time

2,2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) RADICAL SCAVENGING ACTIVITY OF ZPRE

The DPPH radical scavenging activity of ZPRE is presented in Table II. The ZPRE produced

concentration-dependent increase in antioxidant activity. At 400 mg/mL, the antioxidant activity of the ZPRE (45.24%) was significantly ($p < 0.05$) lower when compared with the ascorbic acid (76.61%).

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) OF ZPRE

The ferric reducing antioxidant power of ZPRE is presented in Table III. The ZPRE produced concentration-dependent increase in FRAP value. At 400 mg/mL, the FRAP value of the ZPRE (0.72 µM**)** was significantly ($p < 0.05$) lower when compared with the ascorbic acid (1.90 µM).

ANTIMICROBIAL SENSITIVITY TEST OF ZPRE AGAINST *E. COLI, S. TYPHI* AND *S. AUREUS*

The antimicrobial sensitivity test of ZPRE against *E. coli*, *S. typhi* and *S. aureus* is presented in Table IV. The ZPRE produced concentration-dependent increase in the zone of inhibition against the tested organisms. At 62.5 mg/mL and below concentrations, the zones of inhibition of ZPRE against *E. coli* and *S. aureus* were significantly ($p < 0.05$) lower when compared with the zone of inhibition of ZPRE against *S. typhi*. At 125 mg/mL and above concentrations, there is no significant ($P > 0.05$) difference in the zones of inhibition of ZPRE against *E. coli, S. typhi,* and *S. aureus*. The zones of inhibition of ZPRE against *E. coli, S. typhi,* and *S. aureus* were significantly ($p < 0.05$) lower when compared with the zone of inhibition of ciprofloxacin.

MINIMIUM INHIBITORY CONCENTRATIONS (MIC) OF ZPRE AGAINST ISOLATES

The minimum inhibitory concentration (MIC) of ZPRE against *E. coil, S. aureus* and *S. typhi* were 31.25, 15.63 and 62.50 mg/mL concentration, respectively (Table V).

THE ANTIBIOFILM PRODUCTION ACTIVITY OF ZPRE AGAINST TEST ISOLATES

The result of the antibiofilm production activity of the ZPRE is presented in Table VI. The ZPRE produced significant ($p < 0.05$) concentration-dependent increase in the percentage inhibition of biofilm production in the test isolates. At 7.5 mg/kg, the ZPRE produced 28.87, 34.01 and 66.45% inhibition of biofilm production against *S. typhi*, *E. coli* and *S. aureus*, respectively.

DISCUSSION

This study evaluated the phytochemical composition, antioxidant, antibacterial and antibiofilm producing activities of ZPRE. The ZPRE possesses antioxidant, antibacterial and antibiofilm activities which could be linked to the constituent phytochemicals.

The antioxidant activity of the ZPRE was evaluated using DPPH photometric and FRAP assays. The DPPH is a stable free radical which has an unpaired electron at one atom of nitrogen-bridge and Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay (Sharma & Bhat, 2009). The DPPH in alcohol solution appears purple colour absorbing maximally at 517 nm and changes to yellow with concomitant decrease in absorbance when antioxidant is added (Mishra *et al*., 2012). The ferric-reducing antioxidant power (FRAP) assay measures the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}; here the colorless $[Fe^{3+}$-(2,4,6-Tris(2-pirydyl)-s-triazine)2$]^{3+}$ complex is changed to the intensively blue-colored complex $[Fe^{2+}$-(TPTZ)2$]^{2+}$ in acidic medium and color changes are spectrophotometrically measured at 593 nm (Wojtunik-Kulesza, 2020). The results of the DPPH and FRAP assays suggest that the ZPRE has antioxidant property, which could be linked to the phytoconstituent such as hexadecanoic acid and tetradecanoic acid. The antioxidant property of hexadecanoic acid and tetradecanoic acid have been reported (Bharath *et al*., 2021; Obasi & Ogugua, 2021; Sokmen *et al*., 2014). Antioxidants provide important defense against free radical induced damage, and are critical for maintaining optimum health and wellbeing. Thus, the extract has the potential to retard free radicals induced oxidative stress, which is the fundamental mechanism underlying a number of human cardiovascular, neurologic and other disorders (Sen & Chakraborty, 2011). Antioxidants are also associated with reduction of free radical generation and may be beneficial in recovery of normal cellular functions and treatment of diseases (Sen & Chakraborty, 2011).

Table II: 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity of the ZPRE

	% DPPH radical scavenging activity	
Concentration (µg/ml)	ZPRE	Ascorbic acid
25	7.67 ± 0.24	74.17 ± 0.13
50	10.35 ± 0.15	74.76 ± 0.05
100	22.34 ± 0.24	76.46 ± 0.00
200	30.56 ± 0.25	76.51 ± 0.05
400	45.24 ± 0.23	76.61 ± 0.08

Table III: Ferric Reducing Antioxidant Power values of ZPRE

	FRAP value (µM)	
Concentration (µg/mL)	**ZPRE**	**Ascorbic acid**
25	0.10 ± 0.00	0.16 ± 0.00
50	0.20 ± 0.00	0.37 ± 0.00
100	0.45 ± 0.01	0.85 ± 0.03
200	0.62 ± 0.02	1.25 ± 0.00
400	0.72 ± 0.01	1.90 ± 0.00

The ZPRE produced concentration-dependent increase in zone of inhibition of the bacterial growth, which suggests that ZPRE has concentration-dependent antibacterial activity. The antibacterial activity of the ZPRE could be linked to its phytoconstituents. One of the identified compounds ethyl iso allocholate (a steroid) has been reported in molecular docking study to inhibit the activity of dihydropteroate synthase in *E. coli* (Malathi *et al*., 2017; Muthulakshmi *et al*., 2012). Dihydropteroate synthase (DHPS) is a key enzyme in folate synthesis that catalyzes the condensation of 6-hydroxymethyl-7,8-dihydropteridine pyrophosphate to para-aminobenzoic acid (PABA) to form 7,8-dihydropteroate (Jays *et al*., 2019). The inhibition of dihydropteroate synthase is the mechanism of action of sulphonamides, which possess bacteriostatic action (Cheong *et al*., 2020; Sánchez-Osuna *et al*., 2019). The possible inhibition of dihydropteroate synthase in the test organisms may suggest that ZPRE has bacteriostatic effect. Also, the antibacterial properties of hexadecanoic acid has been reported (Syed *et al*., 2022).

Table IV: Zone of inhibition of ZPRE against the pathogens.

Concentration (mg/ml)	Zone of inhibition (mm) *E. coli*	*S. typhi*	*S. aureus*
ZPRE 7.5	1.87 ± 0.19^{b}	0.20 ± 0.06^{a}	1.93 ± 0.23^{b}
ZPRE 15.5	2.57 ± 0.18^{b}	0.60 ± 0.21^{a}	2.77 ± 0.15^{b}
ZPRE 31.5	3.27 ± 0.18^{b}	1.67 ± 0.24^{a}	3.77 ± 0.15^{c}
ZPRE 62.5	4.90 ± 0.21^{b}	2.77 ± 0.34^{a}	5.73 ± 0.54^{b}
ZPRE 125	8.50 ± 0.29	8.37 ± 0.23	9.00 ± 0.29
ZPRE 250	10.00 ± 0.29	9.50 ± 0.29	10.43 ± 0.23
ZPRE 500	12.27 ± 0.15	12.15 ± 0.08	12.23 ± 0.15
Ciprofloxacin, 7.5 mg/ml	15.00 ± 0.00	15.00 ± 0.00	15.00 ± 0.00

The superscripts "^{abc}" are significantly different ($p < 0.05$) when compared across the row.

Table V: Minimium inhibitory concentrations (MIC) of ZPRE **against isolates**

Extracts	*E. coli*	*S. aureus*	*S. typhi*
ZPRE	31.25 mg/ml	15.63 mg/ml	62.50 mg/ml

Table VI: Percentage (%) inhibition of biofilm production by ZPRE against test isolates

Test compound	Inhibition of biofilm formation (%) *S. typhi*	*E. coli*	*S. aureus*
ZPRE, 7.5 mg/ml	28.87 ± 0.85^{a}	34.01 ± 2.40^{a}	66.45 ± 0.34^{a}
ZPRE, 15 mg/ml	33.89 ± 1.42^{b}	52.01 ± 1.00^{b}	NT
ZPRE, 31.5mg/ml	48.03 ± 1.02^{c}	NT	NT
Ciprofloxacin, 7.5 mg/ml	88.40 ± 0.60^{d}	88.40 ± 0.60^{c}	88.40 ± 0.60^{b}

$^{a,b,c}p < 0.05$ when compared with the ciprofloxacin; NT = Not tested

The MIC value is the lowest concentration of an antibiotic at which bacterial growth is completely inhibited (Kowalska-Krochmal & Dudek-Wicher, 2021). The MIC result indicates that the susceptibility of the organism to the extract was in the order of *S. aureus* > *E. coli* > *S. typhi*. The Gram-positive bacterium (*S. aureus*) was most susceptible while the Gram-negative bacteria (*S. typhi*) was the least susceptible among the tested bacteria organisms to the activity of the extract. This could be attributed to the differences in structure, thickness, and composition of cell membrane and wall of Gram-negative and Gram-positive bacteria. Gram-negative bacteria contain lipopolysaccharides in the cell membrane, which contributes to structural integrity of the membrane, in addition to protecting the membrane from chemical attacks (Domínguez *et al*., 2020). Also, the Gram-negative bacteria has efflux-pumps, which reduce intracellular concentration of drug and institute resistance (Amaral *et al*., 2014). The findings of this study is in agreement with the report of Onwubiko *et al (*2022) on the antibacterial activity of ethanol extracts of *Zapotecca portoricensis* leaf against *Klebsiella pneumonia*, *Pseudomonas aeroginosa*, *S. aureus*, *E. coli* and *Streptococcus pyogenes*.

The antibiofilm activity of the ZPRE was also investigated and the findings suggest that the ZPRE possess concentration-dependent antibiofilm production activity. The antibiofilm effect corroborated the findings of the antibacterial activity. The antibiofilm effect of the ZPRE is most potent against the *S. aureus* and least potent against the *S. typhi*. This is in agreement with report of Sánchez *et al*. (2016), on antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms. The antibiofilm potential of the ZPRE suggest that it could be effective in the treatment of multidrug resistant pathogens. Biofilm is a virulence factor that causes persistence and recurrence of infections; they are extremely resistant to antibiotics and host immune system. Bacteria and other microorganism under the cover of biofilm

exopolysaccharides are up to 1,000 times more resistant to antibiotics than free-floating cells (Sánchez *et al*., 2016).

CONCLUSION

In conclusion, the extract possess antioxidant, antibacterial and antibiofilm production activity which could be linked to the presence of phytocompounds like ethyl iso allocholate and hexadecanoic acid. The study has justified the use of *Zapotecca portoricensis* root in the treatment of bacterial infections by the natives.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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