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Isolation and elucidation of antibacterial compounds from roots and stems of Synadenium glaucescens Pax

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Abstract

Extracts of Synadenium glaucescens Pax are reported to contain biologically active compounds against bacterial and viral infections. This study aimed at isolating pure compounds from its bioactive extracts as well as evaluating their antibacterial efficacy. A phytochemical investigation of the root and stem barks involved total maceration in methanol (MeOH). The root barks extract was then partitioned by Vacuum Liquid Chromatography (VLC) in a solvent gradient system to afford three fractions of Hexane (Hex), Ethyl acetate (EtOAc) and finally MeOH for the least, moderate, and most polar compounds respectively. The MeOH extract of stem barks was also partitioned in the same way by using two solvents: dichloromethane (DCM) and MeOH. All fractions were finally dried on a rotary evaporator at < 60 OC of the water bath. Isolation of pure compound from the EtOAc and DCM led to isolation of four compounds namely, hexacosane (G1), β-sitosterol (G2), octacosyl ferulate (G3) and hexacosanoic acid (G4). Their structures were analyzed and confirmed through NMR, GC-MS and in comparison, with literature. Antibacterial assay for G1, G3 and G4 against P. aeruginosa, E. coli, S. aureus, and E. faecalis was achieved by broth serial microdilution. Compounds, G3 demonstrated strong activity against S. aureus (MIC = 0.125 mg/mL) and weak activity against the rest strains (MIC = 2 mg/ mL). Also, the test results indicated G1 had weak activity against all tested strains (MIC = 2 mg/mL or above). While G4 demonstrated a moderate activity (1.0 mg/mL) against E. coli, S. aureus, E. faecalis and weak against P. aeruginosa (MIC > 2 mg/mL). These findings support traditional use and promise for antibacterial drug agents from S. glaucescens Pax.

Keywords: *antimicrobial, NMR, phytochemistry, ferulic acid, Euphorbiaceae, drug, octacosylferulate*

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Introduction

Antimicrobial resistance is a serious threat against humankind that has fueled research initiatives towards search for new therapeutics (Canli *et al.,* 2017; Nzogong *et al.,* 2018). It is the mission of the World health Organization (WHO) that searching for new medicines be among the interventions towards effective antimicrobial drugs for both preventive and curative measures that protect patients from potentially fatal diseases (WHO, 2015). Despite a limited number of these molecules being evaluated for their suitability for use as drugs (Fabricant and Farnsworth 2001), about 25 % to 50 % have served as the source and inspiration for a large fraction of the current pharmacopoeia (Kingston, 2011). Their diverse structures, forms and concentrations in different organism including plants make them serve a defense mechanism in live organisms (Ostria *et al.,* 2022). These compounds are used either as natural structures or derivative forms (Shelar and Shirote 2011). It is asserted that about 75 % of the drugs for infectious diseases come from natural products (Newman and Cragg 2016) including plants. Infections from resistant bacteria are now too common, and some pathogens have even become resistant to multiple types or classes of antibiotics (Frieden, 2013) thus necessitates a need for new drug agents and sources. *Escherichia coli* is a known versatile and frequently deadly pathogen which is associated with diarrheal diseases (Hunter, 2003), urinary tract infections (UTI) and meningitis (Kaper *et al.,* 2004). Other bacteria such as *P. aeruginosa* is reported to cause pulmonary infections, respiratory insufficiency, UTI and finally morbidity and mortality (Ochoa *et al.,* 2013) while *S. aureus* is known for causing illnesses ranging from mild skin and wound infections to fatal sepsis or multi-organ failure (Chen *et al.,* 2022). *Synadenium glaucescens* Pax (Euphorbiaceae) is a medicinal plant in Tanzania whose crude extracts and pure compounds have been reported for alleviating bacterial and viral infections (Mabiki *et al.,* 2013; Credo *et al.,* 2022). Previous phytochemical screening and isolation experiments on *S. glaucescens* Pax revealed the presence of phenolics, terpenoids, aliphatic hydrocarbons, fatty acids, steroids and more others (Mabiki *et al.,* 2013; Rwegoshora *et al.,* 2022). These groups of compounds are known for their medicinal value in both traditional and modern pharmaceutical industries. However, the number of potential compounds isolated and elucidated from these bioactive sections of this plant is still limited. This study aimed at isolating more pure compounds which are responsible for efficacy of root and stem extracts of this plant.

Materials and methods

Plant collection and Processing

Plant authentication in Njombe region was done by a botanist and the voucher specimen (HOS/FM 3672) was stored in the herbarium of the Department of Botany- University of Dar es Salaam (UDSM). Sample collection was done in Njombe district 1656 m above the see level, (08°34′ to 08°49′ S and 034°55′ to 035°10′ E), in December 2018. The roots (SG2) and stems (SG5) parts were peeled to separate barks and wood parts. The root and stem barks of *S. glaucescens* Pax were air dried in a cold dark room at 15 ^{o}C at the laboratory of Tanzania Tree Seed Agency (TTSA). The conditions were meant to retain the light and temperature sensitive compounds.

Extraction and compounds isolation

Two main samples of root barks and stem barks were involved in the isolation of pure compounds. 1.2 kg of powdered SG stem barks were extracted by total Maceration using methanol (MeOH). For a maximum extraction, filtration was done thrice consecutively after every 72 hours until it showed a clear solution and afforded 185 g of dry extract. 90 g of this extract were pre-adsorbed on silica gel 60 (70-230 mesh ASTM, Merck, KGaA, Darmstadt-Germany). The slurry was partitioned by vacuum liquid chromatography (VLC) in a solvent polarity gradient with yield of 35 g dichloromethane (DCM) and 50 g MeOH extracts. 32.5 g of the DCM fraction were subjected to a column chromatography starting the solvent systems of 100 % PE to 30% MeOH/DCM. After TLC profiling of the eluted vials, a total eleven fractions (*Fs. 1- 11)* were afforded depending on the TLC results. A repetitive clean up *Fs.1, Fs.5* and *Fs.7* by using MeOH yielded compound **G1, G2** and **G3** respectively. The ultraviolet lamp (254 and 365 nm) from Cole-Parmer (800)323-4340, Cat # 97620-41) was used for compound visualization

on TLC. Chemical treatment for visualization of non-conjugated was done by using vanillin (spraying) reagent. A single compound spot on the TLC plate under both treatments (UV and chemical treatment) was used to indicate a pure compound. The melting point (MP) was recorded using a Stuart SMP30 Cole-Parmer machine. The same extraction and partition procedure above) was adopted for the 1.65 kg of powdered root barks with some modification in the extraction solvents. Ethanol (EtOH) was used for the total maceration yielding 98 g dry brown residues while VLC involved a series of Hexane (He), ethyl acetate (EtOAc) and finally EtOH. 85 g of 98 g extract were subjected to VLC. The filtrates were dried using a rotary evaporator at 30 ^{o}C and 60 ^{o}C to afford three (3) crude fractions: 262 mg He (brown-waxy solid), 60 g EtOAc (marigold yellow solid) and 20.1 g EtOH (brown solids). A dry packing of a silica gel column performed using 58 g of EtOAc fraction after multiple elution for establishing suitable solvent systems. A solvent gradient system of 100 % petroleum ether (PE) - 20 % MeOH/ DCM that afforded 155 vials. The vial components were air-dried and profiled on TLC silica gel 60 F_{254}, (Made in Germany, Merck KGaA, 64271 Darmstadt) and the plates treated with vanillin reagent. Basing on the TLC profiles eleven (*fr.1- fr.10*) fractions were afforded. Repetitive purification of *fr.2* yielded **G4**.

Anti-bacterial assay

In vitro antibacterial activity test for compounds G1, G3 and G4 was performed by two-fold serial microdilution method according to the previous described procedure CLSI, 2006 and (Begum *et al.,* 2014; Hiranrat, 2010) with some modifications. These compounds were dissolved in 80 % DMSO v/v to make a stock solution of 4 mg/mL and serially diluted in duplicates for every bacteria strain under test. A standard drug, Gentamicin (Gentakel 10) was used as a positive control while 80 % v/v DMSO served for as a negative control. The representative Gram-negative standard strains of bacteria; *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested while and *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 51559 represented the Gram-positive group. All standard bacteria strains were obtained from the Microbiology Laboratory, Department of Biosciences in the College of Natural and Applied sciences (SUA). They were tested against each compound in duplicate by using a sterile 96-well polystyrene microtitre plate. Two rows of the 96-well microtiter plate were labeled for the negative control, two for the positive control (Gentamicin), two rows for only broth and bacteria. Each well of the plate was loaded with 50 µL of Mueller Hinton broth (MBH) followed by addition of 50 µL DMSO in the negative control rows, 50 µL of Gentamicin, initial concentrations of 1.56 mg/mL for *Enterococcus faecalis* and 0.78 mg/ml for *P. aeruginosa, E. coli and S. aureus* were put in the positive control rows and serially diluted to the last well. 50 µL of 4 mg/ml for each compound in the rows making a total volume of 100 µL. Then 50 µL of the mixture were drawn from the first rows to the subsequent rows until the last ones. The 50 µL from the last wells were discarded. A volume of 50 µL bacteria suspension of 24 hour old culture was adjusted to a density of bacterial cell of approximately 1.5 $\times 10^{8}$ CFU/ mL equivalent to 0.5 McFarland and inoculated into the wells to retain a volume of 100 µL. The plates were incubated at 37 ^{o}C for overnight. The MIC values were recorded visually from the well as the lowest concentration showing no growth. The compounds' efficacy criteria for antibacterial activity was according to previous description by (Mbunde, Mabiki, & Andersson, 2019; Sartoratto *et al.,* 2004) as follows: MIC ≤ 0.5 mg/ml (strong activity), MIC = 0.6-1.5 mg/ml (moderate activity) and MIC > 1.5 mg/ml (weak activity).

Results

Structure elucidation of isolated compounds

Compound **G1,** 173 mg, Melting point: 58- 59.5 ^{o}C reacted purple with vanillin reagent (UV negative). It was isolated as white powder from DCM fraction of the stem bark. Based on both one- and two-dimension spectra, its molecular formula was determined to be $C_{26}H_{54}$, hexacosane. The ^{1}H NMR (CD_2Cl_2, 600 MHz,) spectrum indicated two main signals; at δ 0.88 (6H, t, *J*= 6.8*)* which were characteristic two terminal CH_3 at H-1 and H-26, a second signal at δ 1.26 (48H, *br. s*) was equivalent to protons of twenty-four CH_2 stretch. The ^{13}C NMR (CD_2Cl_2, 150 MHz,) spectrum indicated signals in the shielded region (δ 14.5 – 32.5) which is signal

characteristic for the carbon atoms of saturated hydrocarbon. The important signals at δ 32.5 (C-3 and CH_2, C-24), long signal for CH_2 stretch was observed between 30.2 - 30.3 (C-5 to C-22), 29.9 (C-4 and C-23), 23.3 (C-2 and C-25) and the terminal methyls at 14.5 (CH_3, C-1 and C-26), These spectral data were in agreement with (Aljubiri, *et al.,* 2021; Credo *et al.,* 2022). The COSY spectrum indicated a correlation for the terminal methyls (6H, *t)* with the broad singlet representing $H_3C–CH_2$ connectivity. The HSQC spectral data confirmed the correlation for H-C (1), H-C (26), and the H protons of the of the long CH_2 chain. The HMBC spectrum indicated C- H corrections for H-1 to C-2, C-3 and H-26 to C-25 and C-24. These spectral data and literature helped to conclude the structure of G1 to be hexacosane (Figure 1). To the best of researchers' knowledge, isolation of **G1** (hexacosane) is a first time report in *S. glaucescens* Pax but it was previously reported in *Euphorbia balsamifera* (Aljubiri *et al.,* 2021).

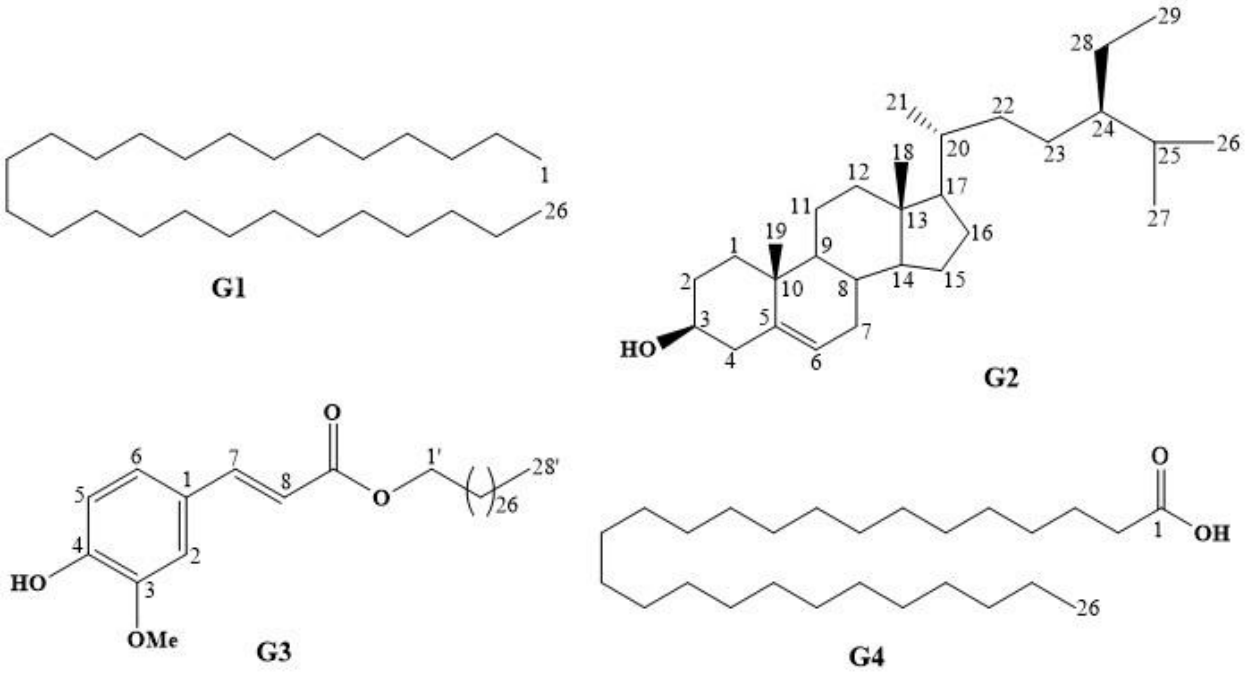

Figure 1. Structures of compounds isolated from S. glaucescens Pax

Compound **G2,** 2 mg was isolated as white crystalline from the DCM fraction of stem barks. The ^{1}H NMR spectrum indicated most protons in the low field region which is a characteristic of tetracyclic skeleton. Two singlets each 3H at δ 0.69 and 1.01 were due to CH_3 for H-18 and H-19. The olefinic proton, H-6 was observed at 5.35 (1H, br d, *J*=5.3) appealing to a characteristic of sterol while a signal at *δ* 3.5 (1H, m) was a proton H-3 of the skeleton. Other evident protons due to secondary methyls were evident at 0.82, 0.84, 0.85, 0.93 and both appeared as doublets *J*= 6.8, 7.5, 6.8 and 6.6 respectively. Its ^{13}C NMR indicated 29 carbon signals which is a characteristic of phytosterols. Two olefinic carbons at *δ* 141.6 (C, C-5) and 122.0 (CH, C-6) while 72.1 was a carbon bearing the OH group (CH, C-3), other carbon signals were 57.4 (C-14), 56.7 (C-17), 50.8 (CH, C-9), 46.5 (CH_2, C-22), 42.9 (C, C-13), 40.4 (CH_2, C-12), 37.9 (CH_2, C-2), 37.1 (CH_2, C-1), 36.7 (C, C-10), 34.5 (CH_3, C-18), 32.5 (CH_2, C-7 and CH, C-20), 32.3 (CH, C-8), 29.8 (CH, C-25), 28.8 (CH_2, C-16), 26.6 (CH_2, C-15), 24.8 (CH_3, C-21), 23.6 (CH_2, C-23), 21.7 (CH_2, C-11), 20.1 (CH_3, C-26), 19.8 (CH_3, C-27), 19.3 (CH_3, C-19), 19.1 (CH_2, C-28), 12.9 (CH, C-24), and 12.3 (CH_3, C-29) as per numbering of the sitosterol skeleton. Based on the DEPT 135 NMR, a total of six CH_3, eleven CH_2, one CH and three quaternary carbon signals were observed. These experimental data were compared and in correlation with (Edilu *et al.,* 2015; Lokadi and Munkombwe, 2015; Nyigo *et al.,* 2016; Ododo *et al.,* 2016; Rwegoshora *et al.,* 2022) together with GC-MS data at m/z 414, **G2** was concluded to be β-sitosterol, $C_{29}H_{50}O$ (Figure 1). Despite its earlier

isolation from leaves (Ododo *et al.,* 2016) and root barks (Rwegoshora *et al.,* 2022), it is for the first time reported from the stem barks of *S. glaucescens* Pax.

Compound **G3** (19 mg), white powder was isolated from DCM fraction of SG stem barks. Its ^{1}H NMR (CD_2Cl_2, 600 MHz) afforded two trans-olefinic protons at *δ* 7.58 (1H, d, *J*= 15.9, H-7), 6.31 (1H, d, *J*=15.9, H-8) and three aromatic protons 6.69 (1H, d, *J*= 8.1, H-6), 7.14 (1H, d, *J*=7.8, H-2), and 6.90 (1H, d, *J*= 7.9, H-5). These ^{1}H NMR data were a characteristic of feruloyl moiety (Kataigiri *et al.,* 1997). It further exhibited signal at δ 5.91 (1H, *s*, -OH), methoxy signal at 3.93 (3H, *s*, -OCH_3), and a methylene at δ 4.15 (2H, t, *J*= 6.7, H-1'), 1.68 (2H, m, H-2'), 1.39 (2H, m, H-3') and a terminal methyl at 0.88 (3H, t, *J*= 6.9, H-28'). A broad singlet (48H) in ^{1}H NMR equivalent to twenty-four CH_2 stretch was observed at *δ* 1.26. The ^{13}C NMR (CD_2Cl_2, 150 MHz) indicated the signal for a carbonyl carbon at 167.6 (C-9), six benzylic carbon signals at 148.5 (C-3), 147.5 (C-4), *δ* 127.7 (C-1), 123.5 (C-6), 115.1 (C-5) and 110.0 (C-2). The olefinic carbons resonated at 144.8 (C-7) and 116.4 (C-8) while 56.6 represented the3-OCH_3). The observed aliphatic chain signals included 65.1 (C -1'). 32.5 (C-19'), 30.2- 30.3 for the CH_2 of C-4' to C-18'), 23.3 (C-27'), 30.4 (C-2'**),** 26.8 (C-3'), and 14.4 for the terminal methyl (C-28'**).** Important HMBC correlations included H-1' to the carbonyl carbon (C-9), and C-2', 3-OCH_3 and OH to C-3, H-2 to C-3, H-7 and H-8 to C-1, H-28' to C-26' and C-27'. These spectral data were compared to and in agreement with (Baldé *et al.*, 1991; Evans *et al.,* 2016; Katagiri *et al.*, 1997; Rwegoshora *et al.,* 2022) and it was assigned as octacosylferulate ($C_{38}H_{66}O_4$) which is also known as erythrinacinate b (Figure 1). Although this compound is reported for the first time from *S. glaucescens,* it was earlier isolated from *Pavetta owariensis* (Katagiri *et al.,* 1997) and *Erythrina caffra* (Baldé *et al.,* 1991). Other closely related compounds including hemicosanylferulate (eicosylferulate) from the root barks (Rwegoshora *et al.,* 2022) and erythrinacinate c from leaves (Nyigo *et al.,* 2022) of *S. glaucescens* have also been isolated.

Compound **G4**, 26 mg was isolated as white powder from the root bark extracts. It reacted purple with vanillin reagent when hot which was an indicator for non-conjugation nature of the C-C skeleton. The ^{1}H NMR (CD_2Cl_2, 600 MHz) indicated four main signals on an aliphatic functionality; δ 2.33 (2H, t, *J*= 7.4) assigned to protons of C-2 which is an α- methlene next to carboxylic acid group (O-CH_2), δ 1.61 (2H, m) due to protons of the β- methylene (C-3) and δ_H 0.88 (3H, t, *J*= 6.9) corresponding to terminal CH_3. A broad singlet observed at δ 1.22- 1.30 (46H) made an equivalence of twenty-three CH_2 chain. The ^{13}C NMR (CD_2Cl_2, 150 MHz,) indicated a single carbon signal in the high filed region at δ_C 175.9 (C, CO_2 C-1) due to carbonyl carbon (C=O) while the rest carbons were in low field region (for aliphatic chain functionality). They included δ 33.8 (CH_2, C-2), 32.5 (CH_2, C-24), broad CH_2 stretch at δ 30.2- 30.4 (CH_2, C_4- C_{24}), 25.3 (CH_2, C-3), 23.3 (CH_2, C-25) while δ 14.4 was a characteristic signal for terminal CH_3. Other CH_2 signals in the ^{13}C NMR included δ 29.6, 29.8 and 29.9 which were also evident in the DEPT135 NMR spectrum. More confirmation of the structure was made using the HSQC spectrum C-H (26), C-H (2), C-H (3) and $(CH_2)_n$ and the HMBC; H-2 to C-1, C-3 and C-3, H-25 to C-24, H-26 to C-23 and C-25. GC-MS, m/z 396, calcd. 396.6899 equivalent to a fatty acid; hexacosanoic acid ($C_{26}H_{52}O_2$). These spectral data were in agreement with (Credo *et al.,* 2022; Nyigo *et al.,* 2022; Rehan *et al.,* 2020; Yamamoto *et al.,* 2015). Based to these spectral and literature information, compound **G4** was assigned to be hexacosanoic acid (Figure 1) which is also known as cerotic acid.

Antibacterial efficacy

Compounds from stem and root barks of *S. glaucescens* demonstrated variable activities against bacteria standard strains. Hexacosane (**G1)** demonstrated weak activity (2 mg/mL) against all three test bacteria strains except on *E. faecalis* which was moderate (1.5 mg/mL). **G3,** octacosylferulate showed strong activity against *S. aureus* (MIC= 0.125 mg/mL), moderate activity against *E. faecalis* (MIC= 1.5 mg/mL), weak activity against *E. coli* (2 mg/mL) and *P. aeruginosa* (> 2 mg/mL). The Weak activity of phenolic compounds against Gram- negative was also reported (Canli *et al.,* 2017). *S. aureus* is arguably among the most problematic of all bacterial pathogens owing in large part to the persistent emergence of antibiotic resistant

strains (Quave *et al.,* 2012). Therefore, the observed activity of this phenolic compound (Octacosylferulate) suggests the possibility of this plant to serve for source of drugs against infections associated with *S. aureus*. Inspite of antibacterial potentials, derivatives of ferulic acid are also reported to be effective antitumor promotors (Akihisa *et al.,* 2000). Compound **G4** (hexacosanoic acid demonstrated moderate activity (1 mg/ mL) against three of the tested strains except *P. aeruginosa* on which it showed weak activity (MIC > 2 mg/mL. These results for hexacosanoic acid are supported by other literatures which indicated that the long chain fatty acid possess bactericidal activity. For example, palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid were found active against *S. pyogenes* and *S. aureus* through inhibition of FabI gene (Zheng *et al.,* 2005). However, it was noted that the standard drug (Gentamicin) was superior of all isolated compounds with MIC values; *S. aureus* (0.003 mg/mL), *E. faecalis* (0.003 mg/mL, *P. aeruginosa* (0.006 mg/mL) and *E. coli* (0.006 mg/mL). Similar result of less susceptibility of Gram negative over Gram positive bacteria to the natural compounds is also reported in EtOAc extracts of *Carpobrotus edulis* (Chokoe *et al.,* 2008). Since the activity of the any drug is associated with its molecular structure *(Duque et al.,* 2018) including the pharmacophore (Duke *et al.,* 2001) and molecular weight (Solvents *et al.,* 2010), a systematic structural modification may improve their activity. Comparing the activities of compounds, there were maximum activities against Gram-positive bacteria (*S. aureus* and *E. faecalis*) due to its antibacterial action against cell wall synthesis (Yamamoto *et al.,* 2015). It is further explained that Gram-positive bacteria have a cell wall composed mostly of peptidoglycan with no protective outer membrane. This structural characteristic makes easy penetration of the toxic phytochemicals into the cells. As opposed to Gram- negative organisms (e.g. *E. coli and P. aeruginosa* in this case) have less peptidoglycan but contain an outer membrane composed of lipopolysaccharide and lipoproteins which make them less permeable (Chokoe *et al.,* 2008). The moderate and weak activities of the compounds against *P. aeruginosa* is associated bacterial biofilm formation favored by the presence of exopolysaccharides (EPS) embedded in an extracellular matrix and to the production of type IV pili, T4P (Ochoa *et al.,* 2013). These results confer with findings that phenolic compounds are among the secondary metabolites in plants that show a wide range of distinct biological activities (Chen *et al.,* 2014; Zhang, *et al.,* 2022).

Conclusion

Isolation of these biologically active compounds; hexacosane (**G1**), β-sitosterol (**G2),** Octacosylferulate (**G3**), (from the stem bark) and hexacosanoic (**G4**) acid from the root barks is reported for the first from *S. glaucescens* Pax. **G3** demonstrated strong antibacterial activity against *S. aureus* while **G1** demonstrated the weakest activities against all tested bacteria strains. The root and stem barks are the potential source of antibacterial agents.

Recommendation

Further studies on structure activity relationship for **G3** are suggested to enhance its efficacy.

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Declarations

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Conflict of interest

The authors declare no conflict of interest.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are included in the Supplementary material but are also available

from the corresponding author on reasonable request.

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