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Isolation and Characterization of Antimicrobial Constituent(s) from the Stem of *Cissus populnea* Guill. & Perr.

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Abstract: *Cissus populnea* Guill. & Perr. (Vitaceae) is used in traditional medicine to treat microbial infections, venereal diseases and infertility, among others. The aim of this research is to isolate and characterize the antimicrobial constituent(s) from the stem of *C. populnea*. The n-butanol fraction of *C. populnea*, being most active, was subjected to silica gel column chromatography, which led to the isolation of white solid and white crystalline substances coded compounds **C1** and **C4C5**, respectively. Spectral analysis (1D and 2D-NMR) of the isolated compounds and comparison with the literature data indicated **C1** to be Bis-(2-ethyloctyl)-phthalate and **C4C5** to be a mixture of stigmasterol and β-sitosterol; **C4C5** exhibited a zone of inhibition ranging from 24 to 29 mm against the test organisms with *Candida albicans* being the most sensitive organism while *Trichophyton rubrum* was the least sensitive organism. Of the standard drugs, ciprofloxacin had 27–37 mm while fluconazole and fulcin exhibited zones of inhibition ranging from 34 mm to 29–32 mm, respectively. The MIC and MBC/MFC values for **C4C5** ranges from 12.5 to 25.0 μg/cm^3 and 25.0 to 50.0 μg/cm^3 against methicillin-resistant *Staphylococcus aureus*, *Staphylococcus. aureus*, *Escherichia coli*, *Candida albicans*, *Trichophyton rubrum* and *Trichophyton mentagrophyte*, respectively. In conclusion, Bis-(-(2-ethyloctyl)-phthalate) and a mixture of stigmasterol and β-sitosterol were identified for the first time from the stem of *C. populnea*.

Keywords: *Cissus populnea*; steroids; phthalates; antimicrobial; stigmasterol; β-sitosterol

Citation: Alex-Asaolu, A.; Uba, A.; Birnin-Yauri, U.A.; Yusuf, A.J. Isolation and Characterization of Antimicrobial Constituent(s) from the Stem of *Cissus populnea* Guill. & Perr. *Drugs Drug Candidates* **2024**, *3*, 172–183. https://doi.org/10.3390/ddc3010010

Academic Editor: Fatma Sezer Senol Deniz

Received: 21 November 2023
Revised: 18 December 2023
Accepted: 26 January 2024
Published: 19 February 2024

1. Introduction

Antimicrobial resistance (AMR) is a global threat that occurs when microorganisms such as fungi, bacteria, parasites and viruses change over the course of time and tends to no longer respond to antimicrobial agents [1,2]. AMR is a public health problem with at least 1.27 million mortalities worldwide and it has the potential to affect people at any stage of life [3]. Complications due to AMR infections that require the use of second- and third-line treatments can lead to serious health conditions such as organ failure as well as prolong care and recovery time, which can last for months [2]. The cause of this threat has been linked to the lack of safe and clean water, misuse and overuse of antimicrobial agents and inadequate infection control, which can encourage the spread of microorganisms that can develop resistance to antimicrobial agents [3]. AMR has substantial economic impact, which, aside from death and disability, include prolonged hospital stays, which increase reliance on costly medications, and financial challenges for those impacted [4]. Thus, there is a need to search for more effective, less expensive and more readily available alternative treatments from natural sources because of their availability and lesser side effects [5].

C. populnea (Figure 1a), belonging to the Vitaceae family, is locally known in Nigeria as *Okoho* by the Idoma and Igala tribes, *Daafaara* or *Latuturwa* by the Hausas and *Ogbolo* or *Ajara* by the Yorubas [6]. The plant is distributed across West Africa from the coast to the Sudan and Sahelian woodlands. Its geographical area spans Senegal, North and South Nigeria, to

Sudan, Uganda and Mozambique [7]. The plant is a woody climbing shrub, 8–10 cm long and 7.5 cm in diameter with a perennial root stock with jointed stems (Figure 1b), often with watery juice. The stock is often an annual rod, drying during the dry season, covering the tree on which it is hung. The bark is cream and smooth when young, then gray and scaly, flaking by a fibrous shell on the old foot. The leaves are alternate, oval and 15–18 cm wide with a slightly pointed apex. The fruit is usually ovoid in shape, smooth and dark purple at maturity. The stems are succulent and sharply quadrangular with sides 6–15 mm wide, constricted at the nodes [8].

(**a**) (**b**)

Figure 1. (**a**) Leaves and ripened fruit of *C. populnea* and (**b**) cross section of *C. populnea* stem. (Source: West African plants: A photo guide); Photographer: Minnattallah Boutros, 2007 at Benin.

C. populnea has been used traditionally for its nutritional value, and its stem has been consumed as food. In recent years, researchers have begun to explore the nutritional value of this plant to better understand its potential contributions to human nutrition. Macronutrient composition: a study conducted by Achikanu and Ani [9] revealed that the stem bark of *C. populnea* is a good source of macronutrients. It contains approximately 1.5% protein, 13.0% fat and 56.0% carbohydrate, making it a good source of energy. *C. populnea* stem bark has also been found to contain a range of vitamins, such as vitamins A, B1, B2, B9, C, D, E, K and B-carotene, that are important for human health. *C. populnea* stems have been found to be a good source of dietary fiber (22.2%), which is important for maintaining healthy digestion and reducing the risk of chronic diseases such as type 2 diabetes and heart disease. *C. populnea* is used in the Niger, Kogi, Benue, Adamawa, Plateau and Kwara states of Nigeria for making vegetable soup for the postnatal stoppage of bleeding [10]. The aqueous extract of the stem bark is used as a fertility enhancer in males in southern Nigeria [11]. A decoction of the stem with native natron is used in northern Nigeria to treat venereal diseases. Preparations from the root are used as an antidote for arrow poisoning and also as a cure for sore breasts [12]. In the Republic of Benin, it is used as a diuretic and in Ghana it is used as a post-harvest ethnobotanic protectant [13]. Extracts from the root of *C. populnea* have been used for the management of skin diseases, boils, infected wounds [14] and for treating urinary tract infections [11].

Phytochemically, Aguoru et al. [15] reported that the stem, root and leaves of the plants contain variable amounts of alkaloids, tannins, anthraquinones, flavonoids and saponins. However, the alkaloid content of the stem was highest with 51.84%, saponin

was highest in the leaf (44.46%) and flavonoid was highest in the root of the plant (43.48%); thus, agreeing with Soladoye and Chukwuma [16], who also reported that saponin was highest in the leaf of *C. populnea*. The stem bark was reported to contain alkaloids, tannins, saponins, flavonoids and terpenoids. Saponin was found to be highest in the stem bark [16]. Bergenin, daucosterol, stigmasterol and β-sitosterol have been isolated from the root of *C. populnea* [17]. Also, Danladi et al. [8] reported the isolation of β-sitosterol from the leaf of *C. populnea* (Figure 2). Essential oil from the stem powder has been reported to have antimicrobial properties [18]. Aqueous extract of the stem bark was reported to possess antioxidant activities [19] and also improves spermatogenesis [11]. The root of C. *populnea* was reported to have anti-sickling [20], anthelminthic [21] and antimicrobial [17] activities. In this paper, we report the isolation and characterization of Bis-(2-ethyloctyl)-phthalate, stigmasterol and β-sitosterol and the evaluation of their antimicrobial activity against some selected microorganisms such as methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*, vancomycin-resistant *enterococcus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *Trichophyton rubrum* and *Trichophyton mentagrophyte*.

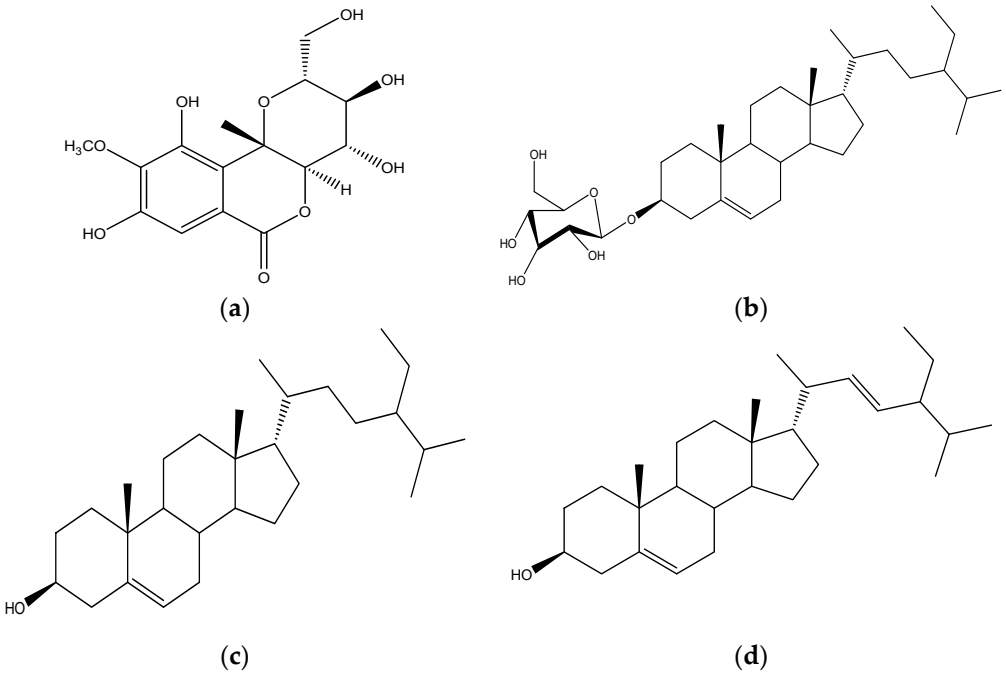


Figure 2. Chemical structures of compounds isolated from leaves and roots of *C. populnea*; (**a**) bergenin; (**b**) daucosterol; (**c**) stigmasterol and (**d**) β-sitosterol. (Source: By Author – Yusuf A. J., drawn using ChemDraw Version 12.0).

2. Results

2.1. Isolation and Characterization of Compounds

2.1.1. Compound **C1**

Compound **C1** was isolated as a white solid compound with a mass of 6.0 mg from fraction B6B7 obtained from silica gel column chromatography of the n-butanol fraction, and it was found to be soluble in chloroform. The identity of the compound was confirmed by comparing its NMR data with the literature, as summarized in Table 1.

Table 1. Summary of 1D- and 2D-NMR Data of **C1** in $CDCl_3$.

Position	^{1}H-NMR	^{13}C-NMR	DEPT	COSY	HMBC
1	0.82	14.3	CH_3	H-2	C-3, C-4, C-5, C-2
2	1.30	21.6	CH_2	H-1	-
3	1.16	45.6	CH_2	-	C-3, C-6, C-10, C-5
4	1.00	24.9	CH_2	H-5	C-3, C-5
5	1.28	22.9	CH_2	H-4	C-6, C-7, C-10, C-4, C-5
6	1.63	29.2	CH_2	H-7, H-8, H-5, H-11	C-9, C-7, C-10, C-4, C-5
7	2.37	32.2	CH	H-6	C-9, C-5, C-7, C-10
8	4.23	68.4	CH_2	H-6	C-7
9	-	178.7	C	-	-
10	1.61	27.3	CH_2	H-11	-
11	0.84	20.9	CH_3	H-10	C-7, C-5
12	-	131.9	C	-	-
13	7.72	129.1	CH	-	-
14	7.54	132.7	CH	-	-

2.1.2. Compound **C4C5**

Compound **C4C5** was obtained as a white crystalline substance with a total mass of 38.0 mg from purification of fraction B6B7 obtained from silica gel column chromatography of the n-butanol fraction and the compound was found to be soluble in chloroform with an uncorrected melting point ranging between 135 and 136 °C. The structure of the compound was confirmed by comparing its NMR data with the literature, as summarized in Table 2.

Table 2. Comparison of 1D-NMR Data of **C4C5** with reported literature.

Position	^{1}H-NMR C4C5	^{1}H-NMR *	^{13}C-NMR C4C5	^{13}C-NMR *	DEPT C4C5
1	1.85	1.85	37.27	37.26	CH2
2	1.46	1.46	31.67	31.67	CH2
3	3.55	3.52	71.83	71.81	CH
4	2.28	2.27	42.31	42.31	CH_2
5	-	-	140.77	140.76	C
6	5.37	5.35	121.72	121.71	CH
7	1.97	1.96	31.92	31.90	CH_2
8	1.49	1.48	-	31.90	CH
9	0.93	0.93	50.15	50.16	CH
10	-	-	36.52	36.51	C
11	1.50	1.49	21.22	21.21	CH_2
12	1.16	1.16	39.70	39.68	CH_2
13	-	-	42.23	42.22	C
14	1.05	1.05	56.88	56.87	CH
15	1.56	1.56	24.37	24.36	CH_2
16	1.71	1.70	28.92	28.92	CH_2
17	1.14	1.13	55.98	55.96	CH
18	0.70	0.69	12.05	12.05	CH_3
19	1.03	1.03	21.09	21.08	CH_3
20	2.02	2.02	40.49	40.49	CH
21	1.00	1.02	23.09	23.07	CH_3
22	5.16	5.10	138.31	138.31	CH
23	5.08	5.03	129.3	129.28	CH
24	1.54	1.53	51.25	51.24	CH
25	1.65	1.65	29.18	29.15	CH_2
26	0.83	0.82	18.99	18.98	CH_3
27	0.79	0.78	19.4	19.40	CH
28	1.17	1.15	25.40	25.40	CH_3
29	0.81	0.80	12.24	12.25	CH_3

* Yusuf et al. [22]

2.2. Antimicrobial Activity of **C4C5**

2.2.1. Susceptibility Test of **C4C5**

The antimicrobial activity of compound **C4C5** and the standard drugs is presented in Table 3; the compound **C4C5** was sensitive to all the test organisms with the exception of vancomycin-resistant *enterococci*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Aspergillus niger* (Table 3).

Table 3. Susceptibility test of **C4C5** and control.

Test Organism	C4C5	Ciprofloxacin	Fluconazole	Fulcin
Methicillin-resistant *Staph aureus*	S	R	R	R
Staphylococcus aureus	S	R	R	R
Vancomycin-resistant *enterococci*	R	S	R	R
Escherichia coli	S	S	R	R
Bacillus subtilis	R	R	R	R
Pseudomonas aeruginosa	R	S	R	R
Candida albicans	S	R	S	R
Aspergillus niger	R	R	R	S
Trichophyton rubrum	S	R	R	S
Trichophyton mentagrophyte	S	R	R	S

KEY: S = Sensitive and R = resistant.

2.2.2. Zone of Inhibition of **C4C5**

Compound **C4C5** exhibited a zone of inhibition ranging from 24 to 29 mm against the test organisms, with *Candida albicans* being the most sensitive organism while *Trichophyton rubrum* was the least sensitive organism. Ciprofloxacin had 27–37 mm, while fluconazole and fulcin exhibited a zone of inhibition ranging from 34 mm to 29–32 mm, respectively (Table 4).

Table 4. Zone of inhibition of **C4C5** and control against the test organism.

Test Organism	Zone of Inhibition (in mm)			
	C4C5	Ciprofloxacin	Fluconazole	Fulcin
Methicillin-resistant *Staph aureus*	27	0	0	0
Staphylococcus aureus	25	0	0	0
Vancomycin-resistant *enterococci*	0	29	0	0
Escherichia coli	28	37	0	0
Bacillus subtilis	0	0	0	0
Pseudomonas aeruginosa	0	27	0	0
Candida albicans	29	0	34	0
Aspergillus niger	0	0	0	29
Trichophyton rubrum	24	0	0	32
Trichophyton mentagrophyte	26	0	0	30

2.2.3. MIC of **C4C5** against the Test Organisms

The MIC value for compound **C4C5** ranges from 12.5 to 25.0 μg/cm^3 against *MRSA*, *S. aureus*, *E. coli*, *C. albicans*, *T. rubrum and T. mentagrophyte* (Table 5).

Table 5. MIC of **C4C5** against the test organism.

Test Organism	Concentration (μg/cm^3)				
	100	**50**	**25**	**12.5**	**6.25**
MRSA	-	-	-	0*	+
Staphylococcus aureus	-	-	0*	+	++
Escherichia coli	-	-	-	0*	+
Candida albicans	-	-	-	0*	+
Trichophyton rubrum	-	-	0*	+	++
Trichophyton mentagrophyte	-	-	0*	+	++

KEY=> - => No turbidity (no growth); 0*=>MIC; + => turbid (light growth); ++ => moderate turbidity. MRSA = Methicillin-resistant *Staph aureus*.

2.2.4. MBC/MFC of **C4C5** against the Test Organisms

The MBC/MFC value for compound **C4C5** ranges from 25.0 to 50.0 μg/cm^3 against MRSA, *S. aureus*, *E. coli*, *C. albicans*, *T. rubrum* and *T. mentagrophyte* (Table 6).

Table 6. MBC/MFC of **C4C5** against the test organism.

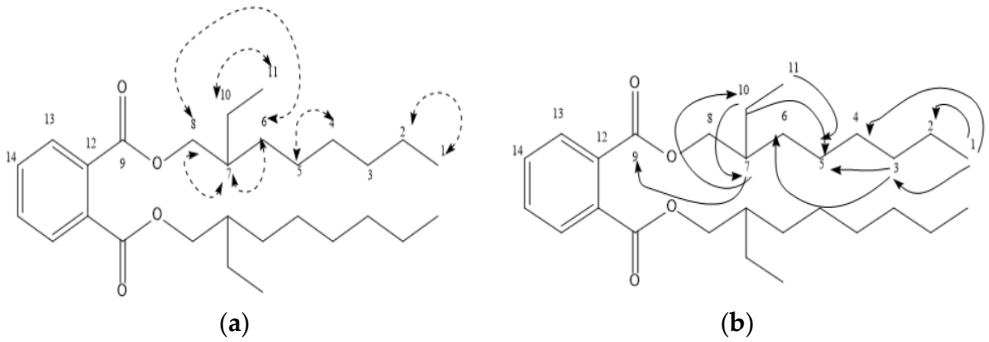
Test Organism	Concentration (μg/cm^3)				
	100	**50**	**25**	**12.5**	**6.25**
MRSA	-	0*	+	+	++
Staphylococcus aureus	-	0*	+	+	++
Escherichia coli	-	-	0*	+	++
Candida albicans	-	-	0*	+	++
Trichophyton rubrum	-	0*	+	+	++
Trichophyton mentagrophyte	-	0*	+	+	++

KEY: - =>No Colony Growth; 0* => MBC/MFC; + =>Scanty colonies growth; ++ => Moderate colonies growth. MRSA = Methicillin-resistant *Staph aureus*.

3. Discussion

Compound **C1** was isolated as a white solid compound with a mass of 6.0 mg from fraction B6B7 obtained from silica gel column chromatography of the n-butanol fraction, and it was found to be soluble in chloroform. The ^{1}H-NMR spectrum of **C1** indicated the presence of aromatic signals at δ_H 7.72 and 7.54 at position 13 and 14, respectively, which is indicative of a substituted aromatic ring [23]. The signal at δ_H 4.23 (H-8) was assigned to the methylene group attached to an electron withdrawing group (ester alcohol) while the signal at δ_H 2.37 was assigned to the methine proton at position 7 (H-7). The spectra further revealed a cluster of multiplet signals upfield, ranging from δ_H 1.00 to 1.63, which were assigned to methylene groups at positions 2, 3, 4, 5, 6 and 8, respectively. Two upfield signals at δ_H 0.82 and δ_H 0.84 were due to terminal methyl groups at positions 1 and 11, respectively. These chemical shift values were similar to those reported for Bis-(2-ethyl hexyl) phthalate [23]. The ^{13}C-NMR and DEPT experiment on **C1** indicated the presence of 14 carbon resonances which are inconsistent with the proton NMR; major resonances observed include δ_C 14.3(C-1), 21.6(C-2), 45.6(C-3), 24.9(C-4), 22.9(C-5), 29.2(C-6), 32.7 (C-71), 27.3 (C-10), 20.9 (C-11), 68.4 (C-8), 178.7 (C-9), 131.9 (C-12), 129.1 (C-13) and 132.7 (C-14). The DEPT-135 revealed the multiplicities of the carbons as two methyl (CH_3), seven methylene (CH_2), three methane (CH) and two quaternary (C) carbons.

The result of the 2D-NMR (H-H-COSY, HSQC and HMBC) confirmed the relationship between the various protons and carbons in the molecule. The HSCQ experiment was used to attach each proton to their respective carbons. The proton at δ_H 7.54 correlated with δ_C 132.7, δ_H 7.72 correlated with δ_C 129.1 and δ_H 4.23 correlated with δ_C 68.4, among others (Table 1). The ^{1}H-^{1}H COSY experiment established the correlations between the protons at H8 (4.23) # H6 (1.63), H7 (2.37) # H6 (1.63), H6 (1.63) # H5 (1.28) and H11 (0.84), H10 (1.61) # H11 (0.84), H2 (1.30) # H1 (0.82) and H5 (1.28) # H4 (1.00), which confirmed the assignment of protons within the oxygenated aliphatic side chain in the molecule (Figure 3a).



(**a**) (**b**)

Figure 3. Some major (**a**) COSY and (**b**) HMBC correlations of **C1**.

The correct assignment of protons, carbons and their linkages in the molecule was confirmed through cross peaks detected on the HMBC spectrum (Figure 3b). Some of the major corrections observed include the long-range correlation between the δ_H 4.23 (H-8) with the carbons at C-5, C-7, C-9 and C-10, which confirmed the attachment of the octyl moiety to the phthalate nucleus as well as the attachment of the ethyl substituent at C7. Similarly, the attachment of the octyl and ethyl moieties to the phthalate nucleus was further confirmed via the long-range correlations between the δ_H 1.63 (H-6) and C4. The correct assignment of the protons and carbons within the octyl side chain was confirmed via the corrections between δ_H 1.28 (H-5) and C-4, C-5, C-6, C-7 and C-10 and δ_H 1.16 (H-3), which correlated with C-3, C-5, C-6 and C-10, among others; the correlation observed between δ_H 0.84 (H-11) and C-5 and C7 further confirmed the attachment of the ethyl side chain at C7. The attachment of the ethyl–octyl moiety to the phthalate nucleus was further substantiated via the correlation observed between δ_H 4.23 (H-8) and C-7 (Table 1). Based on the 1D- and 2D-NMR data of **C1**, and the comparison with related data in the existing literature [23], a tentative structure of **C1** was proposed as Bis-(2-ethyloctyl)-phthalate (Figure 4).

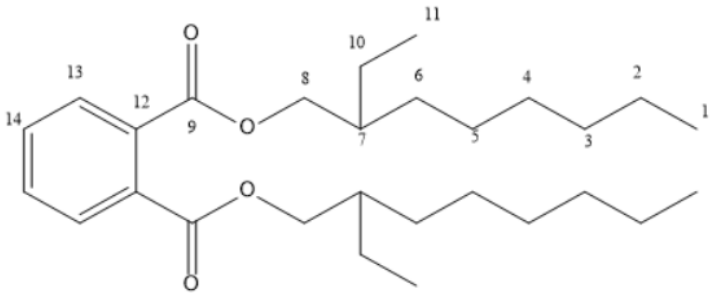


Figure 4. Chemical structure of **C1** (Bis-(2-ethyloctyl)-phthalate).

Compound **C4C5** was obtained as a white crystalline substance with a total mass of 38.0 mg from purification of fraction B6B7 obtained from silica gel column chromatography of the n-butanol fraction and the compound was found to be soluble in chloroform with an uncorrected melting point ranging between 135 and 136 °C, which indicates its purity. The ^{1}H-NMR of **C4C5** indicated the presence of a proton atom of an oxygenated carbon at δ_H 3.55 and a cluster of resonances upfield between δ_H 2.28 and 0.70, thus, suggesting a steroidal nucleus [22,24,25]. The spectrum showed a doublet at δ_H 5.37, which is indicative of a proton at position six (H-6). The spectra further revealed signals at δ_H 0.70 and 1.03, which were assignable to the two tertiary methyl protons at C-18 and C-19, respectively. Two upfield signals at δ_H 0.83 and 1.17 were due to the two methyl groups at C-26 and C-28, respectively. The doublet at δ_H 1.00 was demonstrative of the methyl group at C-21, while the other upfield signal at δ_H 0.81 was due to the methyl group at C-29. Two olefinic protons were clearly observed at δ_H 5.16 and 5.08, which were assigned to C-22 and C-23, respectively, suggesting the compound to be stigmasterol (Yusuf et al., 2015); however, the overlapping signals and the presence of two methylene signals at δ_H 1.31 and 1.09 at C-22

and C-23, respectively, also suggests the presence of β-sitosterol [8]. The carbon-13 and DEPT experiments on **C4C5** indicated the presence of 29 carbon signals, which include six methyl (CH_3), 9 methylene (CH_2), 11 methine (CH) and 3 quaternary (C) carbons. The downfield signals at δ_C 140.77 and 121.72 were assigned to the unsaturated carbons at C-5 and C-6, respectively; and the signals at δ_C 138.31 and 129.3 were also due to olefinic carbons at C-22 and C-23, respectively. The signals at δ_C 12.05 and 21.09 correspond to the angular methyl carbon atoms at δ_C C-18 and C-19, respectively, while the signal at δ_C 71.83 was due to the presence of an electronegative oxygen atom at C-3 [22,24]. Based on the 1D-NMR data and the comparison with related data in the literature (Table 2), the structure of compound **C4C5** was confirmed to be a mixture of stigmasterol and β-sitosterol (Figure 5).

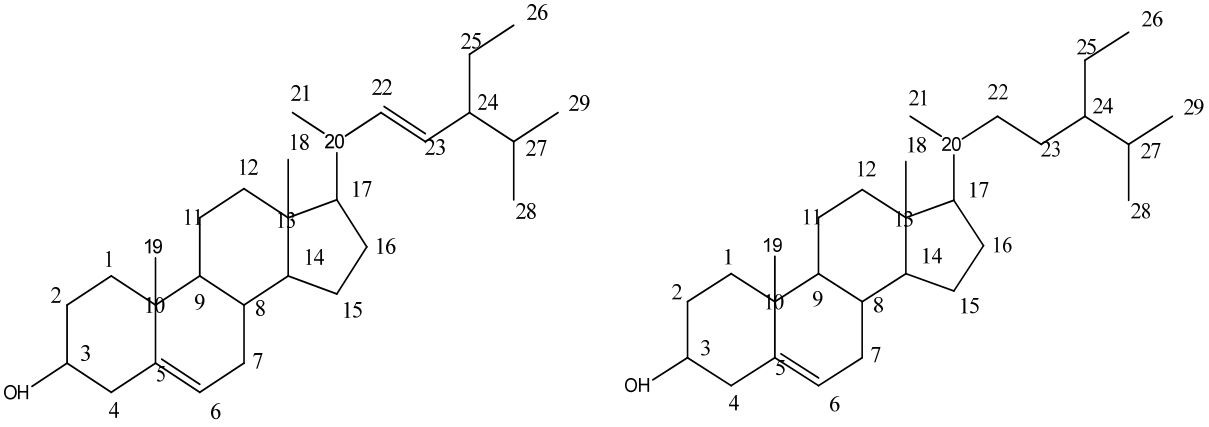


Figure 5. Chemical structure of **C4C5** (stigmasterol and β-sitosterol).

Compound **C4C5** was subjected to antimicrobial screening using agar well and broth dilution techniques, and the findings indicated that the compound exhibited good antimicrobial activity against the test microbes, with favorable MIC and MBC/MFC values. Thus, the compound can be said to have a good broad spectrum of activity considering the mean zone of inhibition diameter is greater than 18 [26,27]. Compounds with MIC values <100 µg/mL are regarded as good antimicrobial agents [28,29]. Thus, the findings of this study were in close agreement with those reported for the antimicrobial activity of stigmasterol and β-sitosterol from the roots of *C. populnea* [17] and β-sitosterol from the leaves of the plant, *C. populnea* [8]. Even though there is limited information on the mechanism of antimicrobial activity of stigmasterol and β-sitosterol, some studies have shown that the compounds have a broad spectrum of antibacterial and antifungal properties [30,31]. Stigmasterol has been reported to inhibit the growth of *C. albicans*, viruses and tropicalis at low concentrations [32]. Studies revealed that the compound may act by inhibiting the activity of sortase, which participate in the pathways involve in the secretion and cell wall anchoring of bacterial virulence factors [31] In addition, Karim et al. [33] and Pratiwi et al. [34] also reported that stigmasterol may act via oxidative stress-induced apoptosis via the Sirtuin family. MRSA is a type of bacteria that is resistant to several antibiotics [1]. It can cause serious health problems such as sepsis, pneumonia and death. Also, *S. aureus*, a gram-positive bacterium, can cause superficial skin lesions, localized abscesses and other infections such as pneumonia, sepsis and toxic shock syndrome [35]. *E. coli* is a causative agent for stomach cramps, bloody diarrhea and vomiting [1]. Likewise, *C. albicans* can cause candidiasis [36]. Compound **C4C5** has demonstrated good activity against these pathogens and, thus, could be studied further for development as an antimicrobial agent.

4. Materials and Methods

4.1. Collection, Identification and Preparation of Plant Material

The plant material of *C. populnea* stem was collected from Ejinya-Eheche in Kogi state, Nigeria. It was identified via taxonomic means at the Herbarium section, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto by Mal. A. Salihu and a voucher specimen number UDUH/ANS/0841 was prepared and deposited. The stems were washed, air dried, ground to fine powder and stored at room temperature for use.

4.2. Extraction and Partitioning of Plant Material

The powdered stem (1.2 kg) was extracted exhaustively by successive maceration using 15 L of 90% methanol for 7 days with constant agitation, and the extract obtained was freed from the solvent by evaporation under pressure with the aid of a rotary evaporator at 40 °C to yield a reddish-brown residue (98.0 g), subsequently referred to as the methanol stem extract (MSE). Some part of the methanol stem extract (90.0 g) was found to be insoluble in water and, thus, was successively washed with n-hexane (1.5 L), chloroform (1 L), ethylacetate (1 L), n-butanol (2 L) and methanol (2L) to afford n-hexane (HFS), chloroform (CFS), ethylacetate (EFS), n-butanol (BFS) and methanol fraction (MFS), respectively.

4.3. Chromatographic Studies

The extract and fractions of *C. populnea* were screened for their antimicrobial activity [37]. BFS, being the most active extract, was subjected to chromatographic studies. The procedure described by Yusuf et al. [22] was adopted for column chromatography. In this method, the n-butanol fraction BFS (3.2 g) was gradiently eluted in a silica gel packed column (5 × 75 cm) using different solvent combinations starting with chloroform 100%, then with mixtures of chloroform: ethylacetate (8:2, 1:1 and 2:8) and then with ethylacetate (100%) to mixtures of ethylacetate: methanol (8:2). An amount of 25 cm^3 each of a total of 231 fractions was collected and combined based on their TLC profiles to give 14 major fractions coded B1-B14. Fractions B6 and B7 were further combined to give B6B7 (0.11 g) based on their TLC profile, which was subjected to further purification with silica gel; mobile phases employed include: hexane (100%), mixtures of hexane: ethylacetate (9:1, 8:2 to 3:7), ethylacetate (100%) and ethylacetate: methanol (9:1). An amount of 20 cm^3 each from a total of 70 collections was made and combined based on their TLC profiles to afford 6 major fractions, **C1**-**C6**. **C1** was found to be a pure compound and C4 and C5 were combined and washed with n-hexane, which afforded compound **C4C5**. Physicochemical properties, solubility test, spectroscopic analysis and antimicrobial activity of **C4C5** were evaluated.

4.4. Antimicrobial Activity of **C4C5**

4.4.1. Microbial Species

The test microorganisms were obtained from stock cultures of the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria. They included methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*, vancomycin-resistant *enterococcus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *Trichophyton rubrum* and *Trichophyton mentagrophyte*. The cultures were maintained on nutrient agar slants for the bacteria species and dextrose agar slants for the fungal species. They were sub-cultured in nutrient broth 24 h prior to testing.

4.4.2. Susceptibility Test

Antimicrobial activity of **C4C5** was determined using agar well diffusion method as described by Yusuf et al. [30]. A quantity (0.001 mg) of **C4C5** was weighed and dissolved in 10 cm^3 of DMSO to obtain a concentration of 100 $\mu g/cm^3$ each. This was the initial concentration used to determine the antimicrobial activity of the compound. Mueller Hinton agar and sabouraud dextrose agar were used as the growth media for the bacteria and fungi, respectively. The media were prepared according to the manufacturer's instructions: they were sterilized at 121 °C for 15 min, poured into the sterile petri dishes and were allowed to

cool and solidify. The solidified medium was seeded with 0.1 cm^3 of standard inoculum of the test microbe; the inoculum was spread evenly over the surface of the medium by the use of a sterile swab. Standard sterile cork-borer of 6 mm in diameter was used to bore a well in the center of each inoculated medium. The wells were filled with 0.1 cm^3 of the solution of the compound and allowed to diffuse for 1 h. Incubation of the inoculated medium was made at 37 °C for 24 h for bacteria and at 30 °C for 1–7 days for fungi. Ciprofloxacin, fulcin and fluconazole discs were used as reference antimicrobials. The tests were conducted in duplicates and the zone of inhibition around the wells was measured in millimeters and used as an assessment of antimicrobial activity.

4.4.3. Minimum Inhibitory Concentration (MIC)

The MIC for each microbial sample was determined using broth dilution technique [30,38]. Mueller Hinton broth and sabouraud dextrose broth were prepared according to the manufacturer's instruction; the medium was dispensed in screw-capped test tubes and sterilized at 121 °C for 15 min and allowed to cool. McFarland's standard turbidity scale number 0.5 was prepared by adding 0.05 cm^3 of barium chloride dehydrate ($BaCl_2 \cdot 2H_2O$) to 9.95 cm^3 of 1% sulphuric acid (H_2SO_4). Normal saline was prepared, and 10 cm^3 was dispensed into sterile test tubes and the test microbe was inoculated and incubated at 37 °C for 6 h. Dilution of the organism suspension was done continuously using sterile normal saline until turbidity matched that of McFarland's scale using visual comparison. Two-fold serial dilution of the extract was performed in the sterile broth to obtain concentrations of 6.25, 12.5, 25.0, 50.0 and 100 $\mu g/cm^3$ for the compound. Having obtained the different concentrations of the samples in the sterile broth, 0.1 cm^3 of the test microbe in the normal saline was then inoculated into the different samples. Incubation was made at 37 °C for 24 h for the bacteria and at 30 °C for 48 h for fungi. The test tubes containing the inoculated broth were observed for turbidity (growth) and the lowest concentration of the compound in the sterile broth which shows no turbidity was recorded as the minimum inhibition concentration [39].

4.4.4. Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

The MBC/MFC was carried out to determine whether the test microbes were killed or whether only their growth was inhibited [30,38]. Mueller Hinton agar and sabouraud dextrose agar were prepared, sterilized at 121 °C for 15 min and transferred into sterile petri dishes to cool and solidify. The contents of the MIC in the serial dilutions was sub-cultured into the prepared media and incubated at 37 °C for 24 h and at 30 °C for 72 h for the bacteria and fungi, respectively. The plates were observed for colony growth; the MBC/MFC was on the plates with the lowest concentration of the compound in serial dilution without colony growth [39,40].

5. Conclusions

Chromatographic separation of the n-butanol fraction of the *C. populnea* stem led to the isolation and characterization of Bis-(-(2-ethyloctyl)-phthalate) and a mixture of stigmasterol and β-sitosterol for the first time. Stigmasterol and β-sitosterol exhibited good antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*, vancomycin-resistant *enterococcus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *Trichophyton rubrum* and *Trichophyton mentagrophyte*. Thus, the plant could be studied further for the isolation of more bioactive compounds.

Author Contributions: Conceptualization, A.A.-A.; methodology, A.A.-A.; software, A.J.Y.; formal analysis, all authors; investigation, A.A.-A.; writing—original draft preparation, A.A.-A.; writing—review and editing. All authors; supervision, A.U., U.A.B.-Y. and A.J.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available in the article.

Acknowledgments: We acknowledged the efforts of Tanko Yohanna of Pharmaceutical Microbiology Laboratory, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto and his team for their assistance in conducting the antimicrobial investigations; Hussaini Ungo-kore for proving the nutrient agar and Patricia Rijo of Universidade Lusofona de Humanidades e Tecnologias, Lisbon, Portugal for her assistance with the NMR analysis.

Conflicts of Interest: The authors declare no conflict of interests.

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