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Original Research Article

Phytochemical Constituents of *Justicia carnea* Leaves and their Antibacterial Activity

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ABSTRACT

The use of plant-based therapeutics in the treatment of bacterial infections is of utmost importance in ethnomedicine and the research into the bioactive constituents of ethnomedicinal plants is still ongoing. Hence, this research aimed to determine the phytochemical constituents of *Justicia carnea* extracts and their antibacterial activities against *Streptococcus pneumoniae, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae,* and *Salmonella typhi*, which are responsible for infections such as urinary tract infection, sepsis, abscesses, pneumonia and diarrhea. The methanol (MCE), n-hexane (HCE), chloroform (CCE), and methanol/water (MWCE) crude extracts were prepared using the pulverized leaves of *J. carnea.* The crude extract was extracted using the column chromatographic technique while purification was achieved using Sephadex LH-20 over flash chromatography. The isolated compounds' purity was assessed using TLC and melting point. 1D and 2D NMR spectra identified chlorogenic acid (**1**), quinaldic acid (**2**), and oleic acid (**3**) as compounds in n-hexane leaves extract. We report for the first time the isolation of quinaldic and chlorogenic acids from *J. carnea*. The antibacterial activities were assessed based on the average diameter of the zone of inhibition, MIC, and MBC. All bacterial assayed were susceptible to MCE ($\geq$ 11.2 $\pm$ 0.12 mm), HCE ($\geq$ 13.1 $\pm$ 0.09 mm), CCE ($\geq$ 9.2 $\pm$ 0.12 mm), MWCE ($\geq$ 10.10 $\pm$ 0.10 mm), chlorogenic acid (**1**) ($\geq$ 11.1 $\pm$ 0.13 mm), quinaldic acid (**2**) ($\geq$ 8.1 $\pm$ 0.07 mm) and not to oleic acid (**3**) ($\leq$ 2.1 $\pm$ 0.07 mm). The results showed that *J. carnea* contained medicinal compounds that are viable antibacterial agents.

Keywords: Ethnomedicine, Antibacterial, Justicia, Chlorogenic acid, Quinaldic acid, Oleic acid.

Introduction

Drugs of plant origin serve as a combination therapy that acts as multidrug resistance modifiers due to their potential biological activities exhibited by their secondary metabolite. The increase in the search for phyto-therapeutic remedies in Nigeria is a consequence of the increased resistance of pathogenic bacteria to chemotherapeutics and antibiotics as well as the availability of phyto-therapeutic remedies. *Justicia carnea* is a member of the *Acanthaceae* family which is an important source of pharmacotherapeutic remedies. *Justicia* is the largest genus of *Acanthaceae* with nearly 600 known species and extracts from the leaf are mostly used in ethnomedicine. [1] Different members of the *Justicia* genus have shown antibacterial activity such as *J. pectoralis* and *J. carnea*, which revealed strong activity against *E. coli.* Different species, extracts, and compounds identified from them have been shown to possess broad-spectrum antibacterial activity. [2, 3] Lignans are the main constituents of active *Justicia*–derived extracts, displaying a wide range of significant clinical attributes however, some compounds isolated from *Justicia* spp. that possess medicinal properties include umbeliferone, Elenoside, Vitexin, Kaempferitrin. [1]

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Sufficient information is not available on the medicinal properties of *J. carnea* leaves extract. [4] Over the years ethnomedicine has been profiled as an alternative source of medicinal therapeutics in the management of arrays of diseases. This is mostly because of the phytochemicals contained in their extractives. [1] Several studies have proven that plant-based pharmaceuticals are potential active agents used in the management and treatments of health-related pathologies, especially resistant bacteria and fungi infections. Hence, many plant-based extracts are in clinical trials for the treatment and management of different ailments. Owing to the resistance of bacterial pathogens, an alternative to the conventional use of orthodox medicine is paramount, and considering the burden of diseases on third-world countries in Africa, especially Nigeria, there is an urgent need to profile extractives of plants known for their antibacterial potentials for use in the management and treatments of diseases caused by bacteria pathogens.

Therefore, in a continued search for novel bioactive compounds from Southeast Nigeria's ethnomedicinal plants, this study aimed at profiling the phytochemical constituents of the leaves extract of *J. carnea* and assessing their ability to inhibit bacterial growth as well as the antibacterial efficacy of its crude extracts prepared using methanol, n-hexane, chloroform, and methanol-water as solvents.

Materials and Methods

General information

The research employed solvents and reagents of analytical grade sourced from Sigma-Aldrich, USA. Ampicillin trihydrate was used as a standard antibacterial drug. Analytical thin layer chromatography (TLC) plates ALUGRAM®Xtra SIL G/UV$_{254}$ (specifications: - layer thickness: 0.20 mm silica gel 60 with fluorescent indicator UVF$_{254/365}$; layer hardness: Standard; soft, plate size: 4*8 cm) was used to evaluate purity. Silica gel 70-230 mesh (63-200 µm) and 230-400 mesh (40-63

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μm) were used for pre-column and column chromatography (CC), respectively.

Sample purification was done using Sephadex LH-20. Chromatogram's visualization was achieved by spraying TLC plates with 10% H_2SO_4 followed by heating. Samples were weighed using analytical weighing balance (OHAUS PX225D, USA), and nuclear magnetic resonance (NMR) spectra data were obtained using an Avance 500 MHz spectrometer (Bruker, USA).

Identification and authentication
In January, 2021, healthy uninfected leaves were collected from bushes around Echeaba in Ebonyi Local Government Area of Ebonyi State, Nigeria. The plant identification and authentication as *Justicia carnea* Lindl was done at the Department of Applied Biology, Ebonyi State University Abakaliki by a taxonomist and labeled EBS/DAB/000012.

Extraction and chromatographic separation
In accordance with increment the in polarity, the successive extraction of the pulverized leaves of *J. carnea* (10 kg) was done using n-hexane, chloroform, methanol, and methanol-water (4:1) as solvents at 24ºC for 48 h with intermittent shaking. The crude extracts were filtered using Whatman filter paper No. 42. At 40 °C the solution's evaporation was achieved under reduced pressure with the aid of a rotary evaporator (Stuart RE 300/MS, UK). A dark green precipitate was obtained for the methanol (116.48 g, 1.16%), n-hexane (90.33 g, 0.90%), chloroform (83.13 g, 0.83%), methanol\water extracts (121.12 g, 1.21%). The entire mass of each extract was dissolved in their respective extracting solvents and then subjected to pre-column chromatographic separation successively with varying proportions of n-hexane, DCM (dichloromethane), MeOH (methanol), and EtOAc (ethyl acetate) mixtures. The derived eluents on concentration gave, 54 g for n-hexane extract, 13 g for methanol extract, 18 g for methanol-water extract, and 22 g for chloroform extract. Eighteen (18) grams of each extracts from n-hexane were subjected to CC. The mobile phase was n-hexane with EtOAc increasing gradient, commencing with 20:0 n-hexane-EtOAc, followed by 16:4, to 12:8, then 5:15 resulting in 20 main fractions. The same TLC profiles indicated a similar fraction, and thus were merged. Fractions 1–10, when merged, gave 10.21 mg, on purification gave chlorogenic acid (**1**, 7.77 mg, 0.0777%) while fractions 11-14 when combined gave 8.2 mg, and subsequent purification gave quinaldic acid (**2**, 5.38 mg, 0.0538%). Then, fractions 15-20 when merged afforded 18 mg; then further purification afforded oleic acid (**3**, 11.41 mg, 0.1141%).

Antibacterial analysis
Bacteria organisms were obtained from Alex Ekwueme Federal University Teaching Hospital Abakaliki. The bacteria were tested for viability by resuscitating them in buffered peptone water; subsequently, they were subcultured into nutrient agar medium and incubated at 37ºC for 24 h then stored at 4ºC until needed. The agar well diffusion technique as described by Ijoma and Ajiwe [5], was employed for the evaluation of the average diameter of the zone of inhibition, MIC, and MBC. A negative and positive control was established using MeOH and ampicillin trihydrate, respectively.

Statistical analysis
The antibacterial data generated by triplicate measurements were presented as mean ± standard error of the mean (SEM). Data analysis employed SPSS version 20 (IBM SPSS Statistic). Groups were examined for significance using Tukey Posthoc (ANOVA) comparison, with significance accepted for $p < 0.05$ (Supplementary data)

Results and Discussion

Spectra Elucidation
Compound **1**: *white powdered solid;* R_f 0.73; mass 7.77 g; Yield 0.0777 %; melting point 206-208ºC [6]. ^{1}H (500MHZ, D_2O, δ, ppm): 7.54 (1H, d, J = 13.5, H-7), 7.01 (1H, d, J = 1.6, H-2), 6.88 (1H, dd, J_1 = 7.5; J_2 = 2.0, H-6), 6.87 (1H, d, J = 7.9,H-5), 6.27 (1H, d, J = 13.5, H-8), 5.34 (1H, m, H-2'), 4.25 (1H, m, H-5'), 3.85 (1H, m, H-4'), 2.22 (2H, m, H-2'), 2.00 (2H, m, H-6'). ^{13}C (500MHZ, D_2O): δ 183.68 (C-7'), 171.87 (C-9), 150.17 (C-7), 148.84 (C-3), 147.08 (C-4), 129.44 (C-1), 125.47 (C-6), 118.89 (C-2), 117.70 (C-5), 117.04 (C-8), 79.62 (C-1'), 75.64 (C-3'), 73.86 (C-4'), 73.48 (C-5'), 41.20 (C-2'), 40.04 (C-6'). NMR spectra of compound **1** matched those of chlorogenic acid (Figure 1) referenced in literature [7] and were thus assigned.
Compound **2**: *Light brown crystalline solid*; R_f 0.77; mass 5.38 g; yield 0.0538 %, melting point 155-156ºC [8]. ^{1}H (500MHZ, D_2O, δ, ppm): 8.05 (1H, dd, J_1 = 9.6, H-8), 8.03 (1H, s, H-4), 7.78 (1H, dd, J_1 = 3.2, H-5), 7.61 (1H, dd, J_1 = 3.2, J_2 = 9.6, H-7), 7.60 (1H, s, H-3), 7.59 (1H, s, H-6). ^{13}C (500MHZ, D_2O): δ 175.78 (C-9), 156.89 (C-2), 148.78 (C-8a), 140.96 (C-4), 133.15 (C-4a), 131.15 (C-8), 130.85 (C-5), 130.62 (C-6), 130.43 (C-7), 123.04 (C-3). NMR spectra of compound **2** matched those of quinaldic acid (Figure 1) referenced in literature [9, 10] thus was assigned.
Compound **3**: *Yellow liquid*; R_f 0.86; mass 11.41 g; yield 0.1141 % melting point 14-16ºC [11]. ^{1}H (500MHZ, MeOH, δ, ppm): 5.39 (1H, m, H-9), 5.38 (1H, m, H-10), 2.29 (1H, t, H-2), 1.99 (1H, s, H-11), 1.55 (1H, m, H-3), 1.29 (2H, s, H-4, H-5, H-6, H-12, H-13, H14, H-17) 1.99 (1H, s, H-8), 1.32 (1H, m, H-7, H-15, H-16), 0.88 (3H, t, H-18). ^{13}C (500MHZ, MeOH): δ 177.60 (C-1), 131.72 (C-10), 131.62 (C-9), 35.11 (C-2), 33.79 (C-16), 33.75 (C-12), 33.22 (C-7), 30.93 (C-14), 30.86 (C-6), 30.75 (C-13), 30.64 (C-15), 30.43 (C-4), 30.40 (C-5), 30.37 (C-11), 30.18 (C-8), 26.25 (C-3), 23.91 (C-17), 14.62 (C-18). NMR spectra for compound **3** were in agreement with literature reports for oleic acid (Figure 1) referenced in literature. [12]

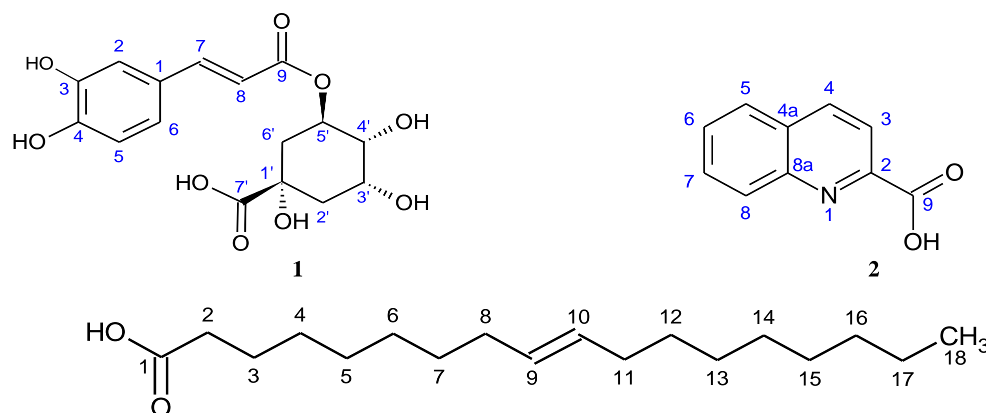

Figure 1: Structure of chlorogenic (**1**), quinaldic (**2**) and oleic (**3**) acids isolated compounds from *Justicia carnea* leaves.

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Antibacterial analysis

Table 1 indicated that compound **3** showed no activity against all bacteria analyzed. According to Oladapo *et al.* [13] zones of inhibition of less than 4.0 mm show resistance, 4.0-12.0 mm indicates intermediates effect, while more than 12.0 mm indicates that the test organism is sensitive to the antimicrobial agent. Therefore, excluding compound **3**, all bacteria assayed were susceptible to studied antibacterial agents. The antibacterial activities of proposed antibacterial agents were significantly different ($p < 0.05$) from ampicillin; however, ampicillin showed more potency than the crude extracts and isolated compounds. Several studies have shown that fatty acids exhibit activity against *E. coli, S. typhi, S. aureus* [14-16] although, poor activity was mostly observed with *E. coli* and *S. aureus*. [17] However, the present study revealed that all assayed bacteria showed resistance to compound **3**. The antibacterial activity of compound **3** in combination with linoleic acid showed synergistic efficacious results, [18] which indicated the potential of compound **3** as a combinatorial therapy in treating bacterial infections. The synergistic attributes of compound **3** were evident in the improved average diameter of zone of inhibition values of HCE, which was ≥ 13.1 ± 0.09 mm. *Escherichia coli* showed the least susceptibility among all bacteria tested against compound **1**; the present study's finding was in line with Mujtaba *et al.* [19] whose work indicated weak inhibitory activity of compound **1** against *E. coli.* Therefore, the improved average diameter of the zone of inhibition of the crude extracts of *J. carnea* was as a result of a synergistic effect. Similarly, the synergistic attributes of compound **1** with Fosfomycin have been studied in silico, [20] and the results equally corroborate the results herein. Table 2 shows the minimum inhibitory concentration (MIC) values of *J. carnea* extracts against assayed bacteria. The MICs of compound **2** showed weak inhibitory activity against *E. coli, S. typhi*, and *S. aureus* at a concentration of 32.000 mg/mL, a similar study by Lee and Lee, [10] validated the results obtained herein. Compound **2** and its derivatives have been reported to show potential antibacterial activity [21] due to the quinoline carboxylic acid at the C-9 position, which makes selective inhibition against some bacterial such as *Clostridium spp.* [10] Hence, this selectivity may likely be the reason for the observed lower inhibitory action of compound **2** against *E. coli, S. typhi*, and *S. aureus*.

Table 1: Average diameter of zone of inhibition values of *J. carnea* against assayed bacteria

Antimicrobial candidates	*Escherichia coli*	*Klebsiella pneumonia*	*Salmonella typhi*	*Streptococcus pneumoniae*	*Staphylococcus aureus*
MeOH	-	-	-	-	-
MCE	18.2 ± 0.12[a]	17.3 ± 0.15[a]	11.2 ± 0.12[a]	20.1 ± 0.07[a]	20.3 ± 0.15[a]
HCE	14.2 ± 0.12[b]	21.1 ± 0.10[b]	13.1 ± 0.09[b]	20.2 ± 0.12[a]	15.4 ± 0.23[b]
CCE	16.1 ± 0.13[c]	22.2 ± 0.09[c]	9.2 ± 0.12[c]	18.2 ± 0.20[c]	18.1 ± 0.06[c]
MWCE	13.0 ± 0.06[d]	15.0 ± 0.09[d]	10.10 ± 0.10[d]	18.1 ± 0.07[c]	16.1 ± 0.07[d]
Compound 1	12.1 ± 0.06[e]	13.1 ± 0.12[e]	11.2 ± 0.09[a]	20.1 ± 0.13[a]	11.1 ± 0.13[e]
Compound 2	8.1 ± 0.07[f]	15.1 ± 0.15[d]	10.0 ± 0.06[d]	15.2 ± 0.12[f]	8.1 ± 0.07[f]
Compound 3	0.0 ± 0.00[g]	0.0 ± 0.00[g]	0.0 ± 0.00[g]	0.0 ± 0.00[g]	2.1 ± 0.07[g]
Ampicillin	19.1 ± 0.12[h]	23.0 ± 0.09[h]	17.2 ± 0.17[h]	28.1 ± 0.12[h]	25.2 ± 0.17[h]

* a, b, c, d, e, f, g, and h indicated the level of significance of the difference between different agents and ampicillin as obtained from SPSS statistics Tukey Posthoc test. Distinct letters in the same column indicated a significant difference ($p < 0.05$) while two letters of the same identity indicated a non-significant difference ($p > 0.05$), - no activity at coverage concentration.

Table 2: Minimum Inhibitory Concentration values of *J. carnea* extracts against assayed bacteria

Antimicrobial candidates	*Escherichia coli*	*Klebsiella pneumonia*	*Salmonella typhi*	*Streptococcus pneumoniae*	*Staphylococcus aureus*
MCE	1.000	2.000	8.000	1.000	0.500
HCE	8.000	1.000	8.000	1.000	4.000
CCE	2.000	0.125	16.000	8.000	2.000
MWCE	8.000	4.000	8.000	8.000	4.000
Compound 1	16.000	32.000	16.000	4.000	16.000
Compound 2	32.000	8.000	32.000	16.000	32.000
Compound 3	-	-	-	-	64.000
Ampicillin	0.250	0.500	1.000	0.250	0.125

MCE methanol crude extract, HCE n-hexane crude extract, CCE chloroform crude extract, MWCE methanol/water crude extract, chlorogenic acid (1), quinaldic acid (2), and oleic acid (3), - no activity at coverage concentration.

Table 3 shows the minimum inhibitory bactericidal (MBC) values of *J. carnea* extracts against assayed bacteria. The MICs and MBCs of compound **1** indicated bacteriostatic and bactericidal activities. Although, crude extracts of *J. carnea* exhibited both bacteriostatic and bactericidal activities against studied bacteria, the therapeutic relevance of bacteriostatic vs. bactericidal action is debatable. [16] As a result, when assessing possible targets for antibacterial activity, the antibacterial attributes (i.e., bacteriostatic or bactericidal) of that peculiar substance cannot be relied upon as a sole basis to exclude its potential attribute as an effective antibacterial agent. [16] The MICs of the crude extracts were greater than the MICs of ampicillin – ampicillin is made up of refined active ingredients.

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Table 3: Minimum Bactericidal Concentration values of *J. carnea* extracts against assayed bacteria

Antimicrobial candidates	*Escherichia coli*	*Klebsiella pneumoniae*	*Salmonella typhi*	*Streptococcus pneumoniae*	*Staphylococcus aureus*
MCE	2.000	4.000	16.000	1.000	1.000
HCE	16.000	2.000	16.000	1.000	8.000
CCE	4.000	0.250	32.000	8.000	4.000
MWCE	16.000	8.000	16.000	8.000	8.000
Compound 1	32.000	32.000	32.000	4.000	32.000
Compound 2	64.000	16.000	-	32.000	64.000
Compound 3	-	-	-	-	-
Ampicillin	0.500	1.000	2.000	1.000	0.250

MCE methanol crude extract, HCE n-hexane crude extract, CCE chloroform crude extract, MWCE methanol/water crude extract, chlorogenic acid (1), quinaldic acid (2), and oleic acid (3), - no activity at coverage concentration

Antibacterial compounds have a different mechanism of action. Compound **1**, for instance, has been shown to increase alteration in outer and plasma membrane permeability, causing functional disturbances, thus resulting in minor nucleotide release. [19, 22, 23] Also, compound **1** has been shown to inhibit the shikimate pathway. [24] Similarly, the antibacterial activity of compound **3** against *S. aureus* was through damaging its cell membrane, [17] while ampicillin disrupts the third and final stage of bacterial cell wall synthesis, followed by cell lysis facilitated by autolytic enzymes associated with cell wall degradation.

Compound **1** in combination with antibiotics has shown improved antibacterial activities against *E. coli* and *S. aureus*; however, Chai *et al.* [25] indicated that compound **1** showed the best antibacterial effect with *S. aureus* at 3000 µg/mL suggesting that the observed antibacterial activities of crude extracts of *J. carnea* was probably due to synergy in their antibacterial bioactivities. Therefore, Considering the trends, emergence, and advances in computer-aided drug design and discovery techniques, the extracts of *J. carnea* can be explored in silico to understand their detailed mechanism of action. [26]

Conclusion

The results of antibacterial activities of compounds **1**-**3** in *Justicia carnea* leaves showed that all bacterial studied were susceptible to crude extracts of *J. carnea* while compounds **1** and **2** showed susceptible to moderate activity relative to the bacterial analyzed, compound **3** was found to show no antibacterial activity. Also, the improved antibacterial activities of the crude extracts were attributed to the synergy of all phytochemicals present in *J. carnea* leaves. Future research should, therefore, provide a more comprehensive and precise interpretation of the interaction between antibiotics and isolated compounds of *J. carnea*. It is of utmost relevance to carry out comparative pharmacokinetic and pharmacodynamic research on compound **1**, compound **2**, and compound **3** and their combination when used together to validate their synergistic attributes in *J. carnea*

Conflict of Interest

The authors declare no conflict of interest

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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