

Antimicrobial activity of stigmasterol from the stem bark of *Neocarya macrophylla*



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Background: Natural products play a significant role in human therapy. They represent a huge reservoir of bioactive chemical diversity and help in understanding the cellular pathways that are essential component of drug discovery process.

Objective: This study was aimed at evaluating the antimicrobial activity of stigmasterol isolated from the stem bark of *Neocarya macrophylla*.

Methods: Stigmasterol previously isolated from the stem bark of *N. macrophylla* was subjected to antimicrobial screening against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), *S. aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Candida albicans* and *Candida krusei* using agar diffusion and broth dilution methods.

Results: Susceptibility test results showed that the compound (100 µg/mL) inhibited the growth of all the test organisms with mean zone of inhibition range from 23 mm to 30 mm except the VRE, *S. typhi* and *K. pneumoniae*. The activity of stigmasterol was compared with that of ciprofloxacin (5 µg/mL), the standard antibacterial drug, and fluconazole (5 µg/mL), the antifungal agent. The test compound displayed a broad-spectrum of activity, and in many cases exhibited comparable antibacterial activity when compared to ciprofloxacin. Interestingly, the compound also showed antifungal activity against *Candida* spp., affording comparable inhibitory effect as fluconazole. The minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC) of stigmasterol range from 6.25 µg/mL to 25 µg/mL and from 12.5 µg/mL to 50 µg/mL, respectively.

Conclusion: These properties suggest that the isolated stigmasterol is a potent and broad-spectrum antibacterial and antifungal agent and as such may serve as a lead compound in the development of novel antimicrobial drugs.

Introduction

Antimicrobial resistance (AMR) driven by inappropriate use of antimicrobial agents has become a global health problem in recent years (Dryden 2017; Laxminarayan et al. 2016; Marston et al. 2016; WHO 2002). The problem of these multidrug resistance and undesirable side effects of most antibiotics necessitate the search for new antimicrobial agents of plant origin. Historically, natural products have been an important source of novel antimicrobial agents and folkloric medicinal use of these products inspired the discovery of several bioactive natural products (Li et al. 2016; Maxson et al. 2016; Simpson et al. 2016). In the last few decades, the interest of the industry in natural products has dwindled; however, in recent times, the need to tackle multidrug resistance and other emerging health problems has fuelled the rebirth of natural product drug discovery. In this regard, natural products may continue to play a vital role in antimicrobial drug discovery; therefore, there is an urgent need for novel natural product scaffolds with superior properties (Chen et al. 2016; Moloney 2016). Nature's biodiversity represents a huge reservoir of bioactive chemical diversity and has been central in the identification and development of therapeutic agents (Siddiqui et al. 2013); hence, drug discovery approaches exploiting nature's chemical diversity may uncover novel natural products with desired bioactivity (Pawar 2014).

Neocarya macrophylla (formerly *Parinari macrophylla*) known as Gawasa in Hausa language is widely distributed along coastal savannahs from Senegal to Liberia, and woody savannahs of

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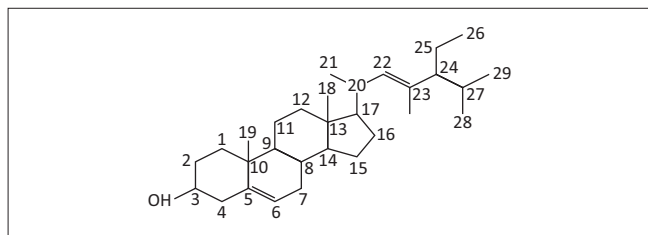


FIGURE 1: Stigmasterol (Stigmast-5, 22-dien-3 β -ol) C₂₉H₄₈O.

Southern Mali, Niger and Northern Nigeria (Abonnier 2004; Burkill 1985). The plant belongs to the Chrysobalanaceae family which is composed of 17 genera, while about 525 species are found in the tropical and subtropical regions (Yakandawala, Morton and Prance 2010). *N. macrophylla* has been used in ethnomedicine for the treatment of a number of ailments and diseases including asthma, dysentery, diarrhoea, skin infections, cancer and ear and eye infections (Warra et al. 2013). The stem bark is extensively used in ethnomedicine in Northern Nigeria in the treatment of pain, inflammation, diarrhoea and snakebites (Amina Yusuf Jega, Department of Pharmaceutical and Medicinal Chemistry, Usmanu Danfodiyo University, Sokoto, Nigeria pers. comm. November, 2012). The antimicrobial activity of the methanol stem bark extract of the plant has been reported (Yusuf et al. 2015b). Recently, isolation and characterisation of stigmasterol (Figure 1) from the stem bark of *N. macrophylla* was reported (Yusuf et al. 2015a). In this study, antimicrobial activity of stigmasterol isolated from the stem bark of *N. macrophylla* investigated against representative gram-positive and gram-negative bacteria and two fungi is reported.

Materials and Methods

Isolation and structure elucidation of stigmasterol

Stigmasterol was previously isolated from the *n*-hexane soluble fraction of the stem bark of *N. macrophylla* as a white crystalline solid substance using a combination of silica gel and sephadex LH-20 column. The structure was established using one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopic analysis and by direct comparison of data obtained with those reported in the literature (Yusuf et al. 2015a).

Antimicrobial activity of stigmasterol

Test organisms

The antimicrobial activity of stigmasterol was determined using selected pathogenic microbes. The microbes were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital Zaria, Kaduna State, Nigeria. All bacterial cultures were checked for purity and maintained in a blood agar slant, while the fungi were maintained on a slant of Sabouraud dextrose agar (SDA). The microbes include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), *S. aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Candida albicans* and *Candida krusei*.

Susceptibility test

The antimicrobial activity of the compound was carried out using stock concentration of 100 μ g/mL. Mueller Hinton agar was used as the growth medium for the microbes. The medium was prepared according to the manufacturer's instructions and sterilised at 121°C for 15 min. It was poured into sterile petri dishes and then allowed to cool and solidify. The sterilised medium was seeded with 0.1 mL of standard inoculum of the test microbe; the inoculum was spread evenly over the surface of the medium using a sterile swab. A standard sterile cork borer of 6 mm diameter was used to bore a well at the centre of each inoculated medium. The wells were filled with 0.1 mL of the solution of the compound and allowed to diffuse for 1 h. Incubation of the inoculated medium was made overnight at 37°C and 25°C for bacteria and fungi, respectively, after which the medium was observed for the zone of inhibition of growth; the tests were conducted in duplicates and the zone of inhibition was measured with a transparent ruler. The mean of the results was recorded in millimetres (mm) (Vollekova, Kostalova and Sochorova 2001).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the compound was determined using the broth dilution method (Vollekova et al. 2001). Twofold serial dilutions of the compound in the sterile broth were made to obtain the concentrations of 100 μ g/mL, 50 μ g/mL, 25 μ g/mL, 12.5 μ g/mL, 6.25 μ g/mL and 3.13 μ g/mL. An amount of 0.1 mL suspension of the standard inoculum of the test microbe was then inoculated into the different concentrations of the compound. The tubes were incubated at 37°C for 24 h and 25°C for 48 h for bacteria and fungi, respectively, after which the plates were observed for turbidity (growth). The MIC was defined as the lowest concentration of the compound inhibiting the visible growth of each micro-organism.

Minimum bactericidal and /fungicidal concentrations

Minimum bactericidal and fungicidal concentration was carried out to determine whether the test microbes were killed or only their growth was inhibited. Mueller Hinton agar broth was prepared, sterilised at 121°C for 15 min and transferred into sterile Petri dishes to cool and solidify. The contents of the MIC in the serial dilution were subcultured into the prepared medium and incubated at 37°C for 24 h; the plates were observed for colony growth; the MBC/MFC was the plate with lowest concentration of the compound in serial dilution without colony growth (Vollekova et al. 2001).

Results

The compound, stigmasterol, exhibited varying degrees of antimicrobial activity against the test microbes. Susceptibility test result showed inhibition ranging from 23 mm to 30 mm against all the organisms, with the exception of VRE, *S. typhimurium* and *K. pneumoniae*. The compound recorded highest sensitivity against MRSA (30 mm) and the least sensitive organism was *P. fluorescens* (23 mm). Ciprofloxacin (5 μ g/mL), the standard antibacterial drug, had inhibitory

effect against all the test organisms except VRE and *P. fluorescens* with zone of inhibition of 32 mm–41 mm, while the standard antifungal drug, fluconazole (5 µg/mL), exhibited activity against the two fungi species tested with inhibition range of 34 mm–35 mm (Table 1).

Discussion

The low MIC value (6.25 µg/mL–25 µg/mL, Table 2) and MBC/MFC (12.5 µg/mL–50 µg/mL, Table 3) indicated that the compound has very good antimicrobial activity against the susceptible organisms considering the fact that compounds with MICs less than 100 µg/mL are regarded as having strong antimicrobial property (Tang et al. 2003). The methanol stem bark extract of *N. macrophylla* (20 mg/mL) was reported to possess good antimicrobial activity against *Streptococcus pyogenes*, *Bacillus subtilis*, *Bacillus cereus*, *E. coli* and *C. albicans*, with inhibition range of 22 mm–34 mm (Yusuf et al. 2015b); the isolated compound from this extract displayed an

improved inhibitory effect and significantly lower MIC value when compared to the extract. The isolation and purification of stigmaterol from the stem bark extract of *N. macrophylla* has therefore allowed for an accurate activity evaluation of the compound, which reveals that the antimicrobial activity observed with the extract might be attributed to its stigmaterol content.

The broad-spectrum antimicrobial activity of stigmaterol observed in this study is in good agreement with previous studies. For example, Abdissa, Legesse and Delelegn (2015) reported the *in vitro* antibacterial activity of stigmaterol isolated from the roots of *Caylusea abyssinica* against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* with zone of inhibition ranging from 11 mm to 18 mm at a higher concentration (50 mg/mL). A similar study conducted on stigmaterol gave a lower zone of inhibition (9 mm–10 mm) against *S. aureus* and *E. coli* at a concentration of 250 µg/mL (Tong-cun et al. 2012). Yinusa et al. (2014) also reported a zone of inhibition of 20 mm–24 mm for stigmaterol (at 50 µg/mL) against *S. aureus* (21 mm), *S. pyogenes* (22 mm), *B. subtilis* (24 mm), *E. coli* (21 mm), *Proteus vulgaris* (21 mm), *S. typhimurium* (21 mm), *S. dysenteriae* (21 mm), *C. albicans* (21 mm), *C. virusei* (20 mm) and *C. tropicalis* (20 mm). This variability in antimicrobial activity observed for stigmaterol could be attributed to the difference in concentration of the compound used in the studies. Kanokmedhakul, Kanokmedhakul and Phatchana (2005) attributed the mechanism of action of steroids to their ability to inhibit bacterial cell surface protein 'sortase', thus preventing transpeptidation. Membrane disruption has been suggested as one of the likely mechanisms of action of sterols on microbes (Tamokou et al. 2011).

Importantly, this study represents the first antimicrobial evaluation of stigmaterol from the stem bark extract of *N. macrophylla* and therefore highlights an important natural source of bioactive stigmaterol in Nigeria. This study has also identified microorganisms susceptible to stigmaterol which were not previously reported. The highest activity (30 mm) exhibited by the compound on MRSA is worthy of mention as MRSA is a substantial public health problem worldwide causing morbidity and mortality and elevated health care costs. It is a leading cause of skin and soft tissue infections in patients reporting to emergency departments for treatment with a rising rate in primary care clinics and intensive care units (Green et al. 2012). MRSA has also been implicated in pneumonia and surgical site infections (Bush 1989; CDCP 2015).

The compound exhibited 29 mm as zone of inhibition against *S. aureus*, which is the most dangerous of all the many common staphylococcal bacteria and has been implicated as a causative agent in skin infections, pneumonia and breast and other fatal infections (Baorto et al. 1994). Enteropathogenic *E. coli* has been implicated in a number of prevalent infectious diseases including diarrhoea, dysentery (Ochoa et al. 2008) and urinary tract infection (Linhares et al. 2013). The good antibacterial activity against *E. coli* (24 mm zone of inhibition)

TABLE 1: Susceptibility of stigmaterol to various test organisms.

Test organism	Mean zone of inhibition (mm)		
	Stigmaterol	Ciprofloxacin	Fluconazole
Methicillin-resistant <i>Staphylococcus aureus</i>	30	32	0
Vancomycin-resistant enterococci	0	0	0
<i>Staphylococcus aureus</i>	29	35	0
<i>Streptococcus faecalis</i>	27	39	0
<i>Escherichia coli</i>	24	37	0
<i>Salmonella typhimurium</i>	0	41	0
<i>Pseudomonas fluorescens</i>	23	0	0
<i>Klebsiella pneumoniae</i>	0	32	0
<i>Candida albicans</i>	25	0	35
<i>Candida krusei</i>	27	0	34

Note: The value 0 represents no activity.

TABLE 2: Minimum inhibitory concentration of stigmaterol on the test organisms.

Test organism	Concentration (µg/mL)					
	100	50	25	12.5	6.25	3.13
Methicillin-resistant <i>Staphylococcus aureus</i>	-	-	-	-	OA	+
<i>Staphylococcus aureus</i>	-	-	-	OA	+	++
<i>Streptococcus faecalis</i>	-	-	-	OA	+	++
<i>Escherichia coli</i>	-	-	OA	+	++	+++
<i>Pseudomonas fluorescens</i>	-	-	OA	+	++	+++
<i>Candida albicans</i>	-	-	OA	+	++	+++
<i>Candida krusei</i>	-	-	-	OA	+	++

-, No turbidity (no growth); OA, minimum inhibitory concentration; +, turbidity (light growth); ++, moderate turbidity; +++, high turbidity.

TABLE 3: Minimum bactericidal and fungicidal concentration of stigmaterol on the test organisms.

Test organism	Concentration (µg/mL)					
	100	50	25	12.5	6.25	3.13
Methicillin-resistant <i>Staphylococcus aureus</i>	-	-	-	OA	+	++
<i>Staphylococcus aureus</i>	-	-	OA	+	++	+++
<i>Streptococcus faecalis</i>	-	-	OA	+	++	+++
<i>Escherichia coli</i>	-	OA	+	++	+++	+++
<i>Pseudomonas fluorescens</i>	-	OA	+	++	+++	+++
<i>Candida albicans</i>	-	OA	+	++	+++	+++
<i>Candida krusei</i>	-	-	OA	+	++	+++

-, No turbidity (no growth); OA, minimum bactericidal and fungicidal concentration; +, turbidity (light growth); ++, moderate turbidity; +++, high turbidity.

displayed by the isolated stigmaterol is hence very encouraging.

Alternative therapy with herbal medicinal products and supplements has continued to increase globally, with a significant proportion of the population in developing countries relying on herbal products for health care needs. In this regard, *N. macrophylla* has been widely used in West Africa for the treatment of a number of diseases including dysentery, diarrhoea and skin, ear and eye infections (Warra et al., 2013). The finding of this study which showed the broad-spectrum antimicrobial activity of stigmaterol isolated from *N. macrophylla* establishes the efficacy of stem bark preparations of *N. macrophylla* in the treatment of those infections. However, further studies are required to address possible safety and toxicity issues with the use of *N. macrophylla*. Analysis of the yields of stigmaterol from stem bark of *N. macrophylla* from different regions and in different seasons should be performed. This may allow the quantification of stigmaterol content in crude extracts, which in turn may provide guidance on the posology of the herbal preparations from this plant.

Conclusion

Stigmaterol isolated from the stem bark of *N. macrophylla* exhibited broad-spectrum antimicrobial activity, highlighting its potential as a candidate in the development of novel antimicrobial drugs. The overall result of this study can be considered as promising with respect to discovering novel drugs from plant sources, especially when the medical importance of the tested microorganisms is considered. The results of this study have established the efficacy of the stem bark preparation of *N. macrophylla* in the management of bacterial and fungal infections.

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Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

Authors' contributions

A.J.Y and M.I.A. designed the research work. M.Y., H.W.A., H.A. and C.O.A. were involved in conducting the research. A.S. and M.M.M. wrote the first draft of the manuscript and all authors were involved in reviewing the final manuscript and finally it was proofread by M.I.A.

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