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# Stigmasterol as Antimicrobial Agent from Root Extracts of *Cola millenii*

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## ABSTRACT

*Cola millenii is a plant found in the North central Nigeria used in the treatment of many diseases like dysenterie, gonorrhoea, skin infection, typhoid fever etc. Phytochemical and antimicrobial studies were carried out on the root part of the plant. The phytochemical screening was carried out using standard procedures and the antimicrobial activity was tested against some pathogens using the tube dilution and agar diffusion method outlined by the National Committee for clinical laboratory studies (NCCLS). The result of the phytochemical screening of n-hexane, ethyl acetate and methanol extracts revealed the presence of cardiac glycoside, saponins, steroids and triterpenes, flavonoids, Tannins and Alkaloids. The results of the antimicrobial activity as indicated by the zone of inhibition of growth of microorganisms ranged from 16mm to 20mm for n-hexane extract, 24mm to 31mm for ethyl acetate extract and 20mm to 27mm for methanol extract. The MIC result for the n-hexane, ethyl acetate and methanol extracts ranged from 7.5mg/ml to 15mg/ml while the MBC result for the extracts ranged from 15mg/ml to 60mg/ml. Extensive chromatographic separation of the ethyl acetate extract (being the most active of all the extracts) led to the isolation of a compound considered to be stigmasterol by spectroscopy. The structure of the compound was established by spectral analysis including 1D and 2D NMR. The antimicrobial activity of the isolated compound as indicated by the zone of inhibition ranged from 26mm to 35mm on staphylococcus aureus, streptococcus pyogenes, E.coli, shigelladysenterie and klebseliapnuemoniea. The antimicrobial results as indicated by the zone of inhibition of the extracts and the isolated substance showed Cola millenii had a fair broad spectrum of activity. The medicinal use of the plant was therefore justified.*

Key words = phytochemicals, antimicrobial, *Cola millenii*, NMR,stigmasterol

## 1.0 INTRODUCTION

Medicinal plants have been shown by many studies as sources of diverse nutrient and non-nutrient compounds. Many of the medicinal plants display antioxidant and antimicrobial properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus it is important to characterize different types of medicinal plants for their antioxidant and antimicrobial potential (Mothana and Lindequist, 2005; Bajpai*et al.*, 2005; Wojdylo*et al.*, 2007). Medicinal plants are known to produce certain bioactive compounds which are responsible for their antimicrobial properties (Rios and Recio, 2005; Sonibare*et al.*, 2009; Kuete*et al.*, 2010; Kuete, 2010). Some classes of chemical compounds that have been isolated from natural products include; terpenoids,

alkaloids, flavonoids and glycosides. The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs. On the other hand, indiscriminate use of commercial antimicrobial drugs in the treatment of infectious diseases has resulted in multiple-drug resistance to many human pathogenic microorganisms. This situation has necessitated a more radical approach in the search for new antimicrobial substances from various sources which could be used as novel antimicrobial chemotherapeutic agents (Navarro *et al.,* 1996) and the resultant difficulty in the treatment of infectious diseases (Tenover, 2006) and also the fact that the number of scientists who are developing new antibacterial agents has dwindled, even as bacteria evolve ever more clever mechanisms of resistance to antibiotics (Krause, 1992). The search for new antibiotics includes various sources such as the synthetic compounds, bioactive agents from aquatic microorganisms, and natural products including medicinal plants. In Africa and other developing countries, it is estimated that 70 to 80% of people rely on traditional healers and herbal practitioners for their health needs (Agyare*et al.,* 2009) and medicinal plants are the main source of remedies used in this therapy. Some of these medicinal plants have been used for the management of several deferent disease conditions such as bacterial infections, parasitic infections, skin diseases, hypertension, pains and inflammation such as rheumatoid arthritis (Muthu*et al.,* 2006).

## 2.0. MATERIAL AND METHODS

### 2.1 Collection, Identification and Preparation of Plant Material

The plant material was collected from the bushes around Makurdi, Benue state, Nigeria in the month of November, 2012. It was authenticated at the herbarium unit by the herbalist of Department of Biological Science, Ahmadu Bello University Zaria-Nigeria; where it was deposited and assigned the specimen voucher number 7. The root was separated from the stem, air-dried for 21days and crushed to coarse powder.

### 2.1 Extraction of Plant Material

500g of the dried powdered root material was macerated successively in n-hexane, ethyl acetate and methanol exhaustively until complete extraction, the solvents were removed *in-vacuo* to yield (7g), (23g) and (34g) of the n-hexane, ethyl acetate and methanol extracts respectively.

### 2.3 Preliminary Phytochemical Screening

A portion of each of the three extracts was subjected to preliminary phytochemical screening using standard methods Trease and Evans, 1987.

### 2.4 Antimicrobial Screening

**Sensitivity Test of the Extract Using Agar Well Diffusion Method**

The disc diffusion method was used (Nostro, 2000). The antimicrobial activities of the n-hexane, ethyl acetate and methanol extracts of the root *Cola millenii* were determined using stock concentration of 100mg/ml.

### 2.5 Determination of Minimum Inhibitory Concentration Using Tube Dilution Method

The minimum inhibitory concentrations of the extracts were determined using the tube dilution method as outlined by the National Committee for Clinical Laboratory Standard (NCCLS). Dilutions (1.57-25mg/ml) of concentration of extract that exhibited sensitivity against the test organisms were prepared in test tubes containing Mueller Hinton broth (MHB). The organisms were inoculated into each tube containing the diluted extracts. The tubes were incubated at 37ºC for 24 hours for bacteria and 48hours for fungi. The lowest concentration in the

series showing no visible growth of the test organisms was considered to be the minimum inhibitory concentration (MIC).

**2.6 Minimum Bactericidal Concentration (MBC)**

The minimum bactericidal concentration of the extract was determined as outlined by the NCCLS on the nutrient agar plates. Minimum bactericidal concentrations were determined by assaying the test tube content of the MIC determinations. A loopful of the content of each tube was inoculated by streaking on a solidified Nutrient agar plate and then incubated at $37^0$C for 24 and 48hours for bacteria after which it was observed for microbial growth. The lowest concentration of the subculture with no growth was considered as minimum bactericidal concentration.

**2.7 Column Chromatography of Ethyl acetate fraction**

7.5g of the Ethyl acetate extract was chromatographed over silica gel packed column of dimension 75 by 3.5cm, the column was eluted continuously using neat n-Hexane, and then n-Hexane: Ethyl acetate mixtures as solvent systems. Seventy five collections, 100ml each were collected. The collections were pooled together based on their TLC profile to give thirteen major fractions and the column was finally washed with methanol to give the fourteenth major fractions.

**2.8 Preparative thin layer chromatography of the Forth Fraction**

Fraction 4 which showed one major spot and one minor spot after properly and carefully washing it with absolute n-hexane was subjected to preparative TLC. Two major spots were got; one was successfully removed as pure compound, and labelled compound DBT. DBT was isolated as a white crystal solid and subjected to spectroscopic analysis to elucidate its chemical structure

**2.9 Physical and chemical test of DBT**

Melting point of the code named compound was determined using melting point apparatus and Liberman-Burchard test was carried out on the compound.

**2.10 Spectroscopic Analysis**

The compound code named DBT was subjected to spectroscopic analysis using NMR for structural elucidation and characterization wit NMR machine of 400 MHz to determine the $^1$H and $^{13}$C spectra of the compound.

**2.11 Sensitivity test of DBT**

The sensitivity test of compound DBT was carried out using the same method used for the crude extracts and with same microorganism

**3.0 RESULT AND DISCUSSION**

The results for the phytochemical screening, antimicrobial activity, spectroscopic analysis and sensitivity test of the extract are shown in Table 1, 2, 3, 4,5 and 6.

**Table 1:** Result of phytochemical analysis

| Phytochemical | n-hexane extract | Ethyl acetate extract | Methanol extract |
|---|---|---|---|
| Anthraquinones | - | - | - |
| Cardiac glycosides | + | + | + |
| Saponins | - | - | + |
| Steroids and terpenes | + | + | + |
| Flavonoids | - | + | + |
| Tannins | - | + | + |
| Alkaloids | - | - | + |

Key: + = Present, - = Absent

**Table 2:** Result of sensitivity as zone of inhibition

| Test organisms | Methanol(mm) | Ethyl acetate(mm) | N-hexane(mm) | Ciprofloxacin 5µg/disc |
|---|---|---|---|---|
| *S.aureus* | 23 | 25 | 20 | 42 |
| *S.pyogenes* | 20 | 26 | 17 | 37 |
| *E.coli* | 24 | 27 | 18 | 35 |
| *S.typhi* | 0 | 0 | 0 | 32 |
| *S.dysenteriae* | 20 | 26 | 16 | 37 |
| *K.pneumoniae* | 27 | 31 | 17 | 47 |
| *N.gonorrhoae* | 21 | 24 | 16 | 0 |
| *S.pneumoniae* | 0 | 0 | 0 | 32 |

**Table 3:** MIC and MBC of the extracts

| Test organisms | MeOH (mg/ml) | EA (mg/ml) | N-hex (mg/ml) | MeOH (mg/ml) | EA (mg/ml) | N-hex (mg/ml) |
|---|---|---|---|---|---|---|
| *S.aureus* | 15 | 15 | 15 | 30 | 30 | 60 |
| *S.pyogenes* | 15 | 15 | 15 | 60 | 30 | 60 |
| *E.coli* | 15 | 7.5 | 15 | 30 | 15 | 60 |
| *S.dysenteriae* | 15 | 15 | 15 | 60 | 30 | 60 |
| *K.pneumonia* | 15 | 15 | 15 | 30 | 15 | 60 |
| *N.gonorrhoae* | 15 | 7.5 | 15 | 60 | 30 | 60 |

**Key**-MeOH= Methanol, EA= Ethyl actate, N-hex= N-hexane

**Table 4:** Result of Chromatography

| Fraction | Eluting solvent | Number of major spots |
|---|---|---|
| 1 | Hexane:ethyl acetate 05:95% | 3 |
| 2 | Hexane:ethyl acetate 10:90% | 2 |
| 3 | Hexane:ethyl acetate 20:80% | 2 |
| 4 | Hexane:ethyl acetate 30:70% | 1 |
| 5 | Methanol | 4 |

**Table 5:** Sensitivity test of the isolate

| **Test organisms** | **Zone of Inhibition(mm)** |
|---|---|
| ***S.aureus*** | 26 |
| ***S.pyogenes*** | 28 |
| ***E.coli*** | 30 |
| ***S.dysenteriae*** | 28 |
| ***K.pneumonia*** | 35 |
| ***N.gonorrhoae*** | 26 |

**Table 6:** Carbon Assignment and Comparison with Standard in Literature

| S/No | position | CHn | $^{13}C$ (ppm) | $^{13}C$ Lit. Prakash, 2012 |
|---|---|---|---|---|
| 1 | **$C_1$** | $CH_2$ | 38.8 | 37.6 |
| 2 | **$C_2$** | $CH_2$ | 29.7 | 32.6 |
| 3 | **$C_3$** | CH | 71.8 | 72.1 |
| 4 | **$C_4$** | $CH_2$ | 45.8 | 42.4 |
| 5 | **$C_5$** | C | 140.8 | 141.1 |
| 6 | **$C_6$** | CH | 121.7 | 121.8 |
| 7 | **$C_7$** | $CH_2$ | 31.7 | 31.8 |
| 8 | **$C_8$** | CH | 31.9 | 31.8 |
| 9 | **$C_9$** | CH | 51.2 | 50.2 |
| 10 | **$C_{10}$** | C | 37.3 | 36.6 |
| 11 | **$C_{11}$** | $CH_2$ | 23.1 | 21.5 |
| 12 | **$C_{12}$** | $CH_2$ | 42.2 | 39.9 |
| 13 | **$C_{13}$** | C | 42.3 | 42.4 |
| 14 | **$C_{14}$** | CH | 56.9 | 56.8 |
| 15 | **$C_{15}$** | $CH_2$ | 26.1 | 24.4 |
| 16 | **$C_{16}$** | $CH_2$ | 29.2 | 29.3 |
| 17 | **$C_{17}$** | CH | 56.8 | 56.2 |
| 18 | **$C_{18}$** | $CH_3$ | 18.8 | 18.9 |
| 19 | **$C_{19}$** | $CH_3$ | 19.8 | 21.7 |
| 20 | **$C_{20}$** | CH | 36.1 | 25.4 |
| 21 | **$C_{21}$** | $CH_3$ | 11.9 | 12.1 |
| 22 | **$C_{22}$** | CH | 33.9 | 46.1 |
| 23 | **$C_{23}$** | CH | 138.3 | 138.7 |
| 24 | **$C_{24}$** | CH | 129.6 | 129.6 |
| 25 | **$C_{25}$** | CH | 36.5 | 29.6 |
| 26 | **$C_{26}$** | $CH_3$ | 21.2 | 20.2 |
| 27 | **$C_{27}$** | $CH_3$ | 21.1 | 19.8 |
| 28 | **$C_{28}$** | $CH_2$ | 39.7 | 40.6 |
| 29 | **$C_{29}$** | $CH_3$ | 12.3 | 12.2 |

**3.1 Result of Isolation of DBT**

A white crystalline solid was obtained from preparative TLC the fourth fraction code name DBT with melting point range of 159-163$^0$C which tested positive to steroids and triterpenes, and was subjected to spectroscopic analysis to elucidate its chemical structure.

**3.2 Discussion**

The result of the phytochemical screening of n-hexane, ethyl acetate and methanol extracts revealed the presence of cardiac glycoside, saponins, steroids and triterpenes, flavonoids, Tannins and Alkaloids Table 1. The results of the antimicrobial activity as indicated by the zone of inhibition of growth of microorganisms ranged from 16mm to 20mm for n-hexane extract, 24mm to 31mm for ethyl acetate extract and 20mm to 27mm for methanol extract in Table 2. The MIC result for the n-hexane, ethyl acetate and methanol extracts ranged from 7.5mg/ml to 15mg/ml while the MBC result for the extracts ranged from 15mg/ml to 60mg/ml Table 3 respectively. The structure of the compound was established by spectral analysis including 1D and 2D NMR and Table 6 showed the spectral comparison of code name DBT compound with that of the literature. From the comparison, it can be seen that there were twenty nine atom signals which comprises of three quartenary,

eleven methine, nine methylene and six methyl Carbon atoms which gave a compound called stigmasterol Fig 1. The antimicrobial activity of the isolated compound as indicated by the zone of inhibition ranged from 26mm to 35mm on *staphylococcus aureus*, *streptococcus pyogenes*, *E.coli*, *shigelladysenterie* and *klebseliapnuemoniea*Table 5. The antimicrobial results as indicated by the zone of inhibition of the extracts and the isolated substance showed that *Cola millenii* had a fair broad spectrum of activity which justified it is traditional uses

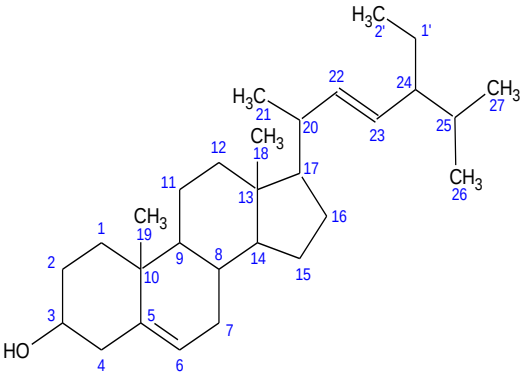



Fig 1: Stigmasterol

## 4.0 CONCLUSION

*Cola millenii*is a plant used in traditional medicine for the treatment of several diseases such as cough, skin infections, gonorrhoea etc. preliminary phytochemical screening revealed the presence of some secondary metabolites and the isolated compounds stigmasterol which are responsible for the observed antimicrobial activity seen against the tested microorganisms. So, the claim made by the local practitioners was verified to be true to some extent.

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