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ANTIMICROBIAL ACTIVITY OF A PLANT ANTHOCYANINE AGAINST SOME HUMAN PATHOGENS

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Abstract

Phytochemicals are secondary metabolites produced by medicinal plants. Some haveshown significant antimicrobial activity to disease causing bacteria's that have developed resistance strains to synthesized drugs. Therefore this investigation is aimed atinvestigating the antimicrobial property and to confirm the growth inhibitory activity of anthocyaninfrom *Phyllanthus muellerianus* plant leaves extract against human pathogen such as*Escherichia coli, Staphylococcus aureus,* and *Salmonella typhi*. Spectroscopic (UV/Vis, FT-IR and 1H NMR) techniques were employed in characterization, while disc diffusion method was used for antimicrobial property and to confirm the growth inhibitory activities.UV-Vis absorption spectrum shows peaks at 313 nm and 482 nm being attributes of anthocyanin. According to FT-IR and 1H NMR investigations the tested sample have cyanidin glycoside and peonidin glycoside. *Phyllanthus muellerianus* plant leaves extractdemonstrated promising activity against the test organisms. The extract exhibits activity on all the test organisms producing zones of inhibition ranging from 11-18mm. The activity was more pronounced (MIC 4.0 mg/mL) against S. typhi while the least activity was against *E. coli* (MIC 50mg/mL). However, *the S. typhi* and *E. coli* were inhibited in all concentration of extract assayed respectively. The minimum inhibition concentration (MIC) showed that the extract had lowest MIC value (4.0 mg/mL) against S. typhi.This investigation therefore supports the traditional medicinal use of P. muellerianus leaves as a source of antimicrobial agents.

Keywords: Antimicrobial activity, Phytochemicals, anthocyanin, cyaniding and peonidin glycosides, *Phyllanthus muellerianus.*

Introduction

Plant phytochemicals such as alkaloids, tannins, steroids, terpenoids and phenolic compounds (Ulubelen, 2003; Cowan, 1999; Akindele and Adeyemi, 2007; Friedman et al., 2008; Balashundram et al., 2004; Abah and Abah, 2010; Chung et al., 1998) has become an increasingly important as an effective antimicrobial agent that can combat strains that are resistant to many conventional drugs (IDSA, 2004; Cox, 1990).

Flavonoids found in fruits, vegetables, nuts, seeds, stems, flowers, tea and wine and propolio (Tagousop et al, 2018; Middleton and Chithan, 1993; Grange and Davey, 1990; Andersen and Markhan, 2006; Chemeaget al., 1993) are ubiquitous in photo-synthesizing cell and display a huge structural diversity based on different rings, aglycone substitutions by OH and OME groups and glycosidation and acylation patterns. Anthocyanins were found to exhibite a potent inhibitory activity against five clinical isolated candida spp (Suket et al., 2014). Anthocyanins and catechinscan regulate the composition of microbes to improve immunity and promote good health

(YongMa et al., 2019; Mahmad et al., 2018).

Phyllanthusmuellerianus is one of the plants of phyllanthaceae and is a small often stunted tropical plant that is found all over West Africa. Other species include *phyllanthusamarus, phyllanthus, sellowianus*(now varieties of *phyllanthusniruri), phyllanthusniruri and phyllanthus simplex* of which phyllanthusniruri has been studied extensively and shown to possess anti-bacterial property (Taylor, 2003).

The plant, *phyllanthusmuellerianus* is a glabrous shrub or woody climber that is widespread in parts of tropical Africa (Hutchinson and Daziel, 1954).

Evaluation of the leaf, stem and root extracts were also found to show good antibacterial activities at high concentrations. Both leaf and bark extracts have been shown to have high antibacterial activity (Doughari and Sunday, 2008; Onocha et al, 2003). This plant is used for gonorrhea, anemia, toothache, paralysis, ophthalmic and added to palm wine for strongly intoxicating (Irvine, 1961) applied on wounds as a dressing, taken for colds and sinusitisand used for throat troubles and glandular fever (Burkill, 1985).

MATERIALS AND METHODS

Plant sample collection and processing

Phyllanthus muellerianus leaves were collected from OyofoOghe inEzeagu Local Government Area, Enugu State, Nigeria and identified by Prof. J.C. Okafor of Department of Applied Biology and Biotechnology, Enugu State University of Science and Technology. The plant materials were air dried indoors at subdued sunlight and ground into powder before extraction of biological active principles.

Extraction of Plant biological active principles

The powdered leaves material (1.5 kg) was percolated in acetone and water (50/50 v/v) at the ratio of 1:3 for 48 hours and filtered. The acetone was distilled out and the aqueous filtrate partitioned successively with n-hexane to remove non polar compounds, chloroform to extract the pigments such as chlorophyll, ethyl acetate for compounds of biological interest such as flavonoids and anthroquinone, and n-butanol heavy hydrocarbons. The fractions were concentrated using rotary evaporator (Model RE—S2CS). (Singh, 2005)).

Column chromatographic separation of biological active principles

The ethyl acetate fraction was eluted with gradient of n-hexane increasing the polarity with ethyl acetate in a column prepared with chloroform and silica gel. Eight fraction were collected and TLC analysis conducted (Adebayo et al., 2005).Fractions with the same R_F value were combined and tested for the presence of flavonoids. Sephadex 20 (HW) was used to purify the sample and eluted with methanol at the rate of 1mL/30 minutes. The fractions so collected were evaporated using rotary evaporator (RE-52CS).

Spectroscopic characterization of biological active principles

Thermo Electron UV spectrometer equipped with vision pro-software's v4.10 was used. Spectroscopic grade chloroform was used as solvent and reference solutions. The fractions of the extract in chloroform (mg/ml) were scanned through UV-visible spectrophotometer (Perkampus, 1995; Espinosa-Morales., 2012)

Different functional groups were determined using FT-IR – 8400S Fourier transform infrared spectrophotometer since different functional groups in a molecule vibrate at distinctly different frequencies. (Adebayo et al., 2007; Adebayo et al., 2009).

The peak and chemical shift of the fractions were determined using NMR (Model Agllent-NMRvnmrs400)to probe the nature and chemical structure of the fractions employing proton (1HNMR) (Claridge, 1999).

Determination of antimicrobial activity

Three clinical isolates (*Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*) were obtained from Microbiology Laboratory, University of Nigeria Teaching Hospital, (UNTH) ItukuOzalla, in Enugu Nigeria. The bacterial species were maintained at 37°C on nutrient agar.

The disc diffusion method was employed according to (Udita et al., 2014; Samiet al., 2017; *Anibijuwon and Udeze, 2009*. Stock solution (100mg/mL) of the extract was prepared. Discs (6mm diameter) were prepared from Whatman filter paper and sterilized at 200°C. The blank sterile disc was placed on the inoculated agar surface and impregnated with 20mL of stock solution. An antibiotic disc of Traflox (20µg) was used as control. The plates were incubated at 37°C for 24h. All tests were performed in triplet and the antimicrobial activity was expressed as the mean diameters of clear zones of inhibition.

Minimum inhibitory concentration (MIC)

Minimum inhibition concentration was carried out using broth dilution method as previously reported by Eloff (1998). Dilutions (25 – 40mg/mL) of concentrations of extract of P.muellerianus plant leaves that exhibited sensitivity against the tested organisms were prepared using test tubes containing 9mL of the concentrations. The test tubes were inoculated with (0.3mL) suspension of the standard inoculated and incubated at 37°C for 24h. The lowest concentration of extracts which show no visible growth of the broth was taken as the minimum inhibition concentration.

RESULTS AND DISCUSSIONS

The UV/visible absorption at 313 and 482 nm (Fig-2) is within the range 315-325 and isattributed to the absorption from the B-ring cinnanoylsystem (Thaler, 1999). The FTIR spectra are presented in Fig. 1 and Table 1. The stretching vibration ofalcoholic and phenolic group's containing C-H, and COof aromatic compounds occurs between the bands at 1300 – 1000 while stretching vibrations between 1600-1300 cm-l represents C-O and C-C stretchingvibrations of glucoside bonds. Theabsorption at 1512 cm-1 therefore are due tostretching and contraction of all of the bonds in the ringof a hetero aromatic compound. This is region (1500- 1520 cm-1) of double bonds of which carbonyl group isthe most important that give the very strong intensity in their IR spectra and represents presence of carbonyl group (C=O) from aldehydes andketones (Baciu et al., 2013).

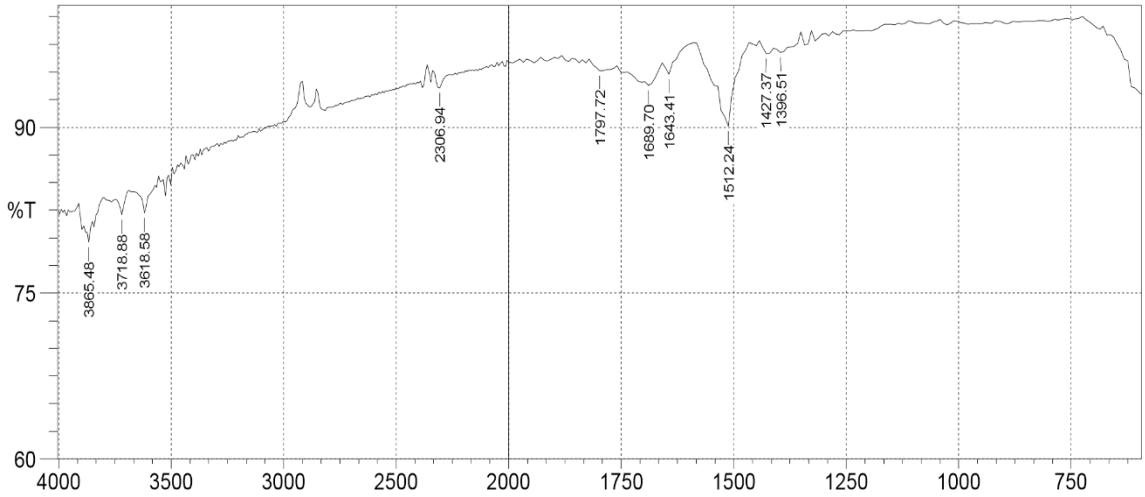

Fig.1: FTIR analysis result of purified *P.muellerianus* plant leaves extract

The absorption at 1797 cm−1 is anattribute of overtone and combination of aromaticcompounds with a high intensity (Suart, 2004; Baciu et al., 2013). The absorption at 3618.58 cm−1 is a stretching vibrations of OH groups and presence of absorption at1300-1000 cm-1 represents the presence of stretching vibration of alcoholic and phenolic group's containing C-O vibration of aromatic compounds (Ning, 2011; Baciu et al., 2013) confirming that phenols is in the aromatic ring of the sample. The presence of absorption at 3618 cm-1 in the sampleis an attribute of methoxyl group (-OCH3) and it suggests a peonidin anthocyanin (Espinosa-Morales et al., 2012).

Table 1: IR spectra data result of purified P.muellerianus plant leaves extract

No.	Peak	Intensity	Assignment
1	1396.51	96.774	C-O and C-C stretching
2	1427.37	96.647	C—O Str.
3	1512.24	90.104	C=C Bending (Aromatic)
4	1211.34	94.791	C—O Str
5	1689.7	93.777	C=O Str.
6	1797.72	95.089	C=O (overtone and combination bands of
7	2306.94	93.551	aromatic compounds
8	3618.58	82.275	C=C Accumulated double bonds
9	3718.88	82.065	—OCH_3 (para position) methoxyl group (-
10	3865.48	79.587	OCH_3)X—H (X = H, O, S etc.) stretching vibrations

The spectra of the extract are shown in Fig-2. The spectra of the sample are suggestive of the presence of an anthocyanin, with aromatic signal in the 6-9 ppm region (Tulio et al., 2007 and 2008). The signal in sample (8.6, 8.5 and 8.3 ppm) could be due to the presence of aromatic protons of an anthocyanin.

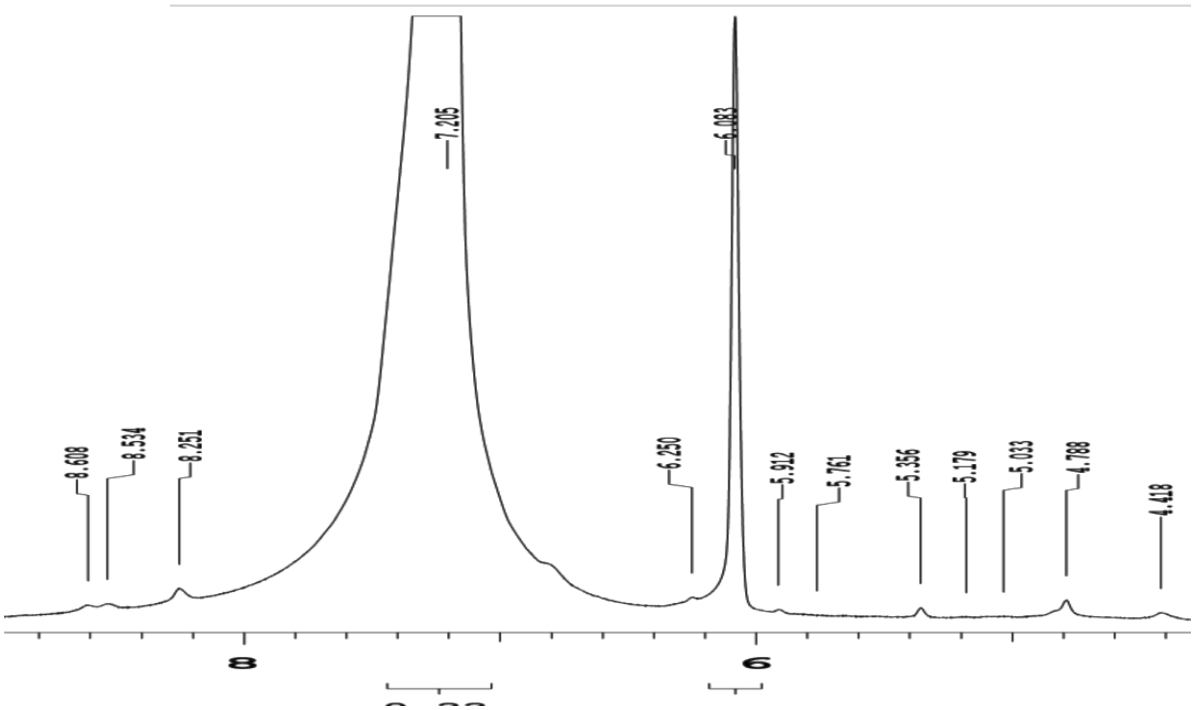

Fig. 2: Result of ^{1}H NMR of purifiedP.muellerianus plant leaves extract

The 1H NMR spectra of the extract is dominated by the resonances from cyanidin 3- glycosides (cy 3 – gly). The signal at 8.3 ppm represent proton at carbon atom number 6 of cyanidinaglycone while 8.6 ppm are assigned to carbon atom number 4 of cyaniding aglycone. It can then be concluded that sample contain cyanidin 3 – glycoside.

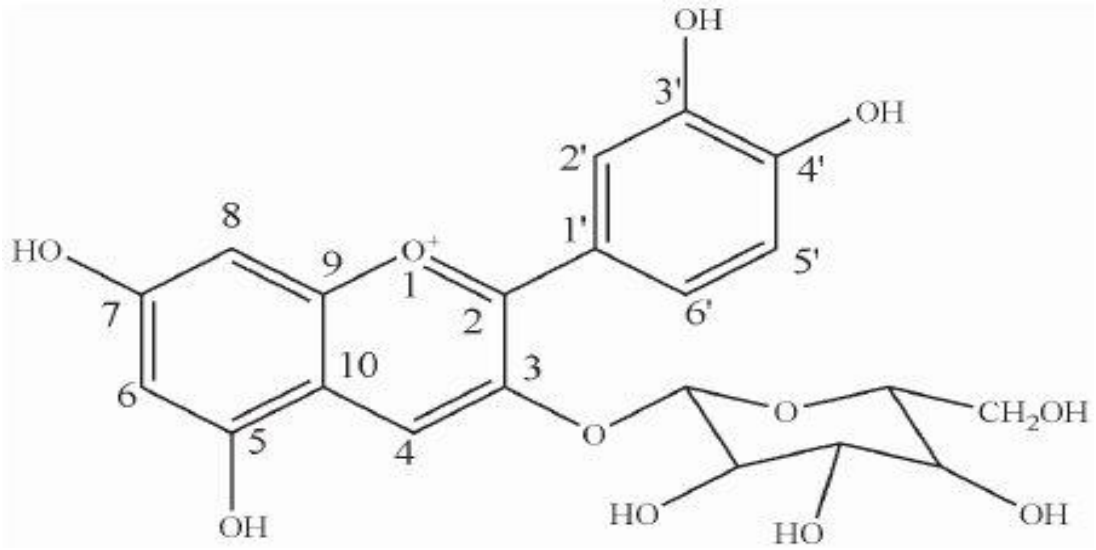

Fig. 3: Chemical structure of cyanidin 3-dioxalyglucoside

The P. muellerianus flavonoid extracts exhibits activity on all the test organisms producing zone of inhibition, ranging from 9 – 16mm (Table 2). The results of the minimum inhibition concentration (MIC) of the cold water and ethanol extracts varied with the types of microorganism assayed. The most significant activity was observed with the extract (MIC 4.0 mg/mL) against S. typhi while the least activity was against E. coli of MIC 50mg/mL.

The growth of S. typli was inhibited in all concentration of extract assayed. This agrees with the athnobotanical findings that the plant was effective in the treatment of several aliments including constipation, flu and typhoid fever caused by S. typhi in the Bahamas (Jonas and Kenneth, 1995). The minimum inhibitory concentration (MIC) values of the leaf extract against tested organisms were shown in Table 3. The MIC values were 50, 12.5, and 4 mg/mL for leaf extract against the tested organisms. The MIC value against the tested gram – positive bacteria 12.5mg/mL and against gram-negative bacterial ranged from 4.0 – 50mg/mL. Antimicrobial potency of the plant extracts against these organisms expressed in MIC indicates that the extract inhibit S. typhi at 4 mg/mL and S. aureus at 12.5 mg/mL concentrations and the least susceptible concentration was 50mg/mL for *E. coli*.

Table 2: Antimicrobial activity of Cold water and Ethanol extract of P. muellerianus leaves

Diameter of Zone Inhibition (mm) of*Phyllanthus muellerianus* plantextract (mg/disc)						
Test Organism	**400**	**200**	**100**	**50**	**25**	**Traflox**
Escherichiava Coli	16 + 0.3	12 + 0.0	9 + 0.7	9 + 0.1	9 + 0.4	36 + 0.0
Staphyloccus aureus	16 + 0.1	14 + 0.0	9 + 0.5	9 + 0.0	9 + 0.1	40 + 0.5
Salmonella typhi	16 + 0.7	15 + 0.3	9 + 0.0	9 + 0.4	9 + 0.3	40 + 0.3

Table 3: Minimum Inhibitory Concentration (MIC) of Cold water and ethanol extract of Phyallanthusmuellerianus leaves

Test organisms	**MIC values of ethanol extract (mg/mL)**	**MIC values of Traflox (mg/mL)**
Escherichia Coli	50	2
Staphylococcus aureas	12.5	4
Salmonella typhi	4	3

CONCLUSION

A characteristic 1H NMR spectra was obtained for the purified*Phyllanthus muellerianus* plant leaves extract. The analysis revealed the presence of signal at 8.6, 8.5 and 8.3 ppm. They are probably due to the presence of aromatic protons of cyanidin glycoside.It was concluded that the purified*P. muellerianus* leaves extract demonstrated a strong activity against the microorganisms used. This investigation confirms the used in the folk medicine and a source of antimicrobial substance for possible treatment of many diseases caused by microorganisms. However, the mechanism of action of *P. muellerianous* leaves extract was not investigated.

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