The Pharmaceutical and Chemical Journal, **2018, 5(1):117-122**

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Preliminary Report on the Isolation and Phytochemical Evaluation of Antimicrobial Constituents from the Leaves of *Nturukpa*- A Common Nigerian Herb (*Pterocarpus santalinoides* DC)

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Abstract The aim of this study was to isolate and evaluate the antimicrobial constituent of the leaves of *Pterocarpus santalinoides* DC (Fabaceae) growing in the tropical rain forest region of Nigeria, Sub Saharan Africa. The powdered leaf materials were defatted with n-hexane and extracted in a soxhlet extractor with 90.0 % methanol. The methanol extract was further fractionated to yield the chloroform, ethyl acetate, butanol and methanol soluble fractions. Each of the fractions was screened for antimicrobial activity using Agar-well diffusion method. Phytochemical tests were carried out using standard procedures. The fractions that had antimicrobial activity were subjected to column chromatographic separation and monitored by analytical thin layer chromatography (TLC). The n-butanol fraction that gave the best antimicrobial bioassay result was subjected to further fractionation and purification to afford some pooled fractions.FractionF5 that showed good antimicrobial activity was subjected to semi-preparative chromatographic purification to isolate the bioactive constituent. The isolate was characterized using a combination of analytical and spectral techniques which included: ultra violet (UV), Fourier Transform Infrared spectrometry (FTIR), gas chromatography-mass spectroscopy (GC-MS), etc.

Preliminary phytochemical screening showed that the leaves of *Pterocarpus santalinoides* contained alkaloids, tannins, flavanoids, saponins, terpenoids and free anthraquinones. All fractions tested showed mild antimicrobial activity. The Infrared (IR) chromatogram revealed peaks that strongly suggest the presence of O-H, C-H, RC=C-H, C=O and C=N functional groups. These preliminary results indicated that the antimicrobial potential of leaves of *Pterocarpus santalinoides* may be attributable to such hydroxyl (OH)-bearing constituents like alkaloids, tannins, saponins, terpenoids, flavanoids and anthraquinones which might be responsible for this activity either singly or in combinations. Other analytical and phytochemical parameters are being evaluated to elucidate the actual structure of the active ingredient.

Keywords Antimicrobial, *Pterocarpus santalinoides,* Phytochemical, Fourier Transform Infrared Spectrometry

Introduction

Pterocarpus santalinoides DC (Fabaceae) is a tropical monoecious flowering tree that is widely distributed in Nigeria as well as other tropical countries- Brazil, Cameroon, Ghana and Senegal. It is commonly called *nturukpa* by the Igbos, *gunduru* by the Hausas and *gbengbe* by the Yorubas [1]. It has proven to be a plant of many applications: Its leaves are eaten as vegetable and are used as fodder for livestock, tannins and dyes obtained from the bark are used for dyeing and the tree provides good windbreak around settled areas and farms. The plant has been employed as an ornamental tree and its poles have been used for fencing [1]. In South Eastern Nigeria, the leaves are used to treat gastro-intestinal diseases, diabetic syndrome and are known to exhibit antipyretic activity

[2]. The leaves are also used for treatment of skin diseases [3]. In North Central Nigeria, the leaves are used in treatment of pain and inflammation of lower abdomen, stomach ache and other infectious diseases [4]. In India, fruit extracts are used traditionally in treating headache, skin diseases, boils, fevers etc. while the stem bark extracts were reported to have antibacterial, anti-diabetic and hepatoprotective activities [5].

Microbes are dominating the earth and influencing almost all spheres of life. For us humans to regain hygienic control of our environment, we need new combative agents that are cheap, less harmful to us and as effective as previous agents; perhaps even more effective with the advantage of being less familiar to these organisms [6, 7]. Following the widespread use of this plant in traditional medicine to treat mixed infections, a need arises to assess the antimicrobial activity of the leaves of *Pterocarpus santalinoides* and characterize the constituents responsible for such effect. Thus, this study was an attempt at assessing the reported antimicrobial activity of the leaves of *Pterocarpus santalinoides* growing in the tropical rain forest region of Nigeria, Sub Saharan Africa, isolation of antimicrobial constituents from fractions of the leaf extract and evaluation of the isolated compounds. We present in this report the preliminary findings from the bioassay-guided evaluation of the antimicrobial constituents of the plant and the isolation of the bioactive constituent.

General Experimental Procedures

The non-flowering part of *Pterocarpus santalinoides* (Fabaceae) was collected from Owerri Imo State, South-East Nigeria in 2016 and duly identified by a botanist. A sample of the plant with herbarium number-UPH/P/081 and Reference No- UPH/PSB/2016/005 was preserved for future reference.

The leaves were plucked off the branches, dusted, cleaned and air-dried for 14 days at room temperature before they were milled to coarse powder. The powdered materials were de-fatted in batches while their crude alcohol extracts were obtained from the dried marc by Soxhlet extraction using 90% methanol. The dried methanol extract was reconstituted in 30ml of methanol and distilled water added to make up to 150ml. The sequential partitioning was done using chloroform, ethyl acetate and n-butanol. All fractions were concentrated using rotary evaporator before drying over the water bath at 40°C.The pulverized leaves, methanol extract and the chloroform, ethyl acetate, n-butanol and aqueous fractions were screened for phytoconstituents associated with anti-microbial effects following standard procedures [8, 9].

Anti-microbial Evaluation

The methanol, n-butanol and aqueous fractions found to have the most phytochemical constituents were evaluated for antimicrobial activity against *Staphylococcus aureus* (Gram positive organism) *Escherichia coli* (Gram negative organism) and *Candida albicans* (fungi)using the agar well diffusion technique. An inoculum of an overnight broth culture of test organism (compared to McFarland standard-1.5 x 10^{8}cfu/ml) was mixed by swirling with molten Mueller-Hinton agar, poured and transferred to a sterile Petri dish. A 6mm diameter well was cut in the plate after solidification using a cork borer and 0.1ml solution of each fraction prepared with protic solvent dimethylsulfoxide-DMSO (concentration 50mg/ml-0.25g of fraction in 5ml DMSO) was introduced into the well. The agar plate was incubated (37°C for 24 h for bacteria and 27°C for 48 hours for fungi) and observed for zones of inhibition. Solutions of ciprofloxacin and fluconazole were used as positive controls while an antibiotic-free agar plate served as negative control.

Determination of Minimum Inhibitory Concentration (MIC) of N-butanol Fraction

Stock solution of the most active fraction (n-butanol;50mg/ml) was serially diluted two folds in Mueller-Hinton broth to obtain decreasing concentrations. An aliquot of overnight broth culture of test organism in sterile normal saline was introduced into each fraction dilution. The mixtures in sterile test tubes were incubated and observed for turbidity. Ciprofloxacin and fluconazole were used as postive controls as standard antibacterial and antifungal agents while normal saline was used as a negative control.

Determination of Minimum Bactericidal/Fungicidal Concentration of N-butanol Fraction

From each of the MIC tubes that showed no growth a loopful was removed and inoculated onto antibiotic-free Mueller-Hinton agar plate, incubated and inspected for presence of colonies.

Column Chromatography of Active N-Butanol Fraction

The n-butanol fraction was introduced into a column chromatogram wet packed with slurry of silica gel (60-120 mesh size) and n-hexane by mixing the fraction with dry silica gel. Column chromatography was conducted using continuous gradient elution technique with n-hexane, chloroform and methanol as eluting solvents. The several fractions obtained from the column were developed using thin layer chromatography employing pre-coated silica gel TLC plates as a way of monitoring the separation.

Spectral Analysis of Fraction F5

Concentrated Fraction F5 was reconstituted in 5ml methanol, transferred to the sample bottle, concentrated and subjected to physicochemical analytical techniques such as Fourier transform infrared spectrometry (FTIR) and ultraviolet spectrometry in order to characterize the compound.

- **Fourier Transform Infrared Spectrometry:** The viscous sample was placed between two sodium chloride pellets, fixed in the cell and measured. A background spectrum was obtained by converting an interferogram to a frequency data by inverse Fourier Transform. Next a single beam spectrum of the sample was collected and the ratio between single beam sample spectrum and the single beam background spectrum gives the spectrum of Fraction F5.

Results and Discussion

The result of the phytochemical screening (Table 1) showed that the milled leaves were rich in phytochemicals (alkaloids, terpenoids, anthraquinones, tannins, flavanoids and saponins) with well documented antimicrobial effect [10, 11]. The methanol and n-butanol fractions are the richest in terms of phyto-constituents with the other fractions following in this order- Aqueous>Ethyl acetate>Chloroform>Hexane.

Table 1: Phytochemical Screening Results for Fractions/Extracts

Constituents \ Fractions/Extracts	Milled Leaves	Hexane	Chloroform	Ethyl Acetate	n-butanol	Methanol	Aqueous
Alkaloid							
Dragendorff's test	+	-	-	-	+	+	+
Hager's test	+	-	-	-	+	+	-
Tannin	+	-	+	+	+	+	+
Flavonoid	+	-	-	+	+	+	+
Saponin	+	-	-	-	+	+	+
Terpenoids	+	-	-	+	+	+	+
Free anthraquinone	+	-	-	-	+	+	-

Key: + = Present; **-** = Absent

All fractions evaluated for antimicrobial activity showed considerable effects against test organisms (Gram-positive *Staphylococcus aureus,* Gram-negative *Escherichia coli* and Fungus *Candida albicans*) when compared to standard agents-ciprofloxacin (antibacterial) and fluconazole (antifungal) at 50mg/ml. *Escherichia coli* showed resistance to the methanol fraction (Table 2).

Table 2: Inhibition Zone Diameter (Mm) Exhibited by Fractions/Extracts (50 mg/ml)

Fractions	Methanol	n-butanol	Aqueous	Ciprofloxacin	Fluconazole	DMSO
Test Organisms						
Staphylococcus aureus	10mm	18mm	14mm	37mm	-	11mm
Escherichia coli	nil	12mm	11mm	17mm	-	nil
Candida albicans	11mm	13mm	12mm	-	23mm	nil

Key: **-** = Antimicrobial drug not tested against organism

The solvent (dimethylsulfoxide-DMSO) used to reconstitute extracts showed intrinsic antimicrobial activity against *Staphylococcus aureus.* Minimum inhibitory concentration (MIC) studies on the n-butanol fraction showed that it

has an MIC value of 6.25mg/ml for *Staphylococcus aureus* and 12.5mg/ml for *Escherichia coli and Candida albicans.* The n-butanol fraction also proved to be bactericidal and fungicidal with minimum bactericidal/fungicidal concentration value at 25mg/ml (Tables 3 & 4).

Table 3 Minimum Inhibitory Concentration of n-butanol Fraction

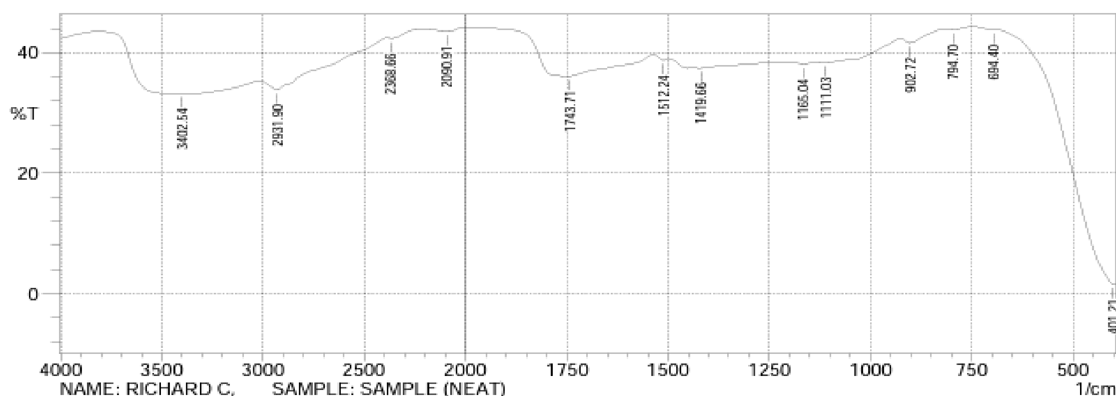
Test Organisms	**Concentration of n-butanol fraction (mg/ml)**				
	50	**25**	**12.5**	**6.25**	**3.125**
Staphylococcus aureus	-	-	-	-	+
Escherichia coli	-	-	-	+	+
Candida albicans	-	-	-	+	+

Key: **-** = No growth; + = Growth

Table 4: Minimum Bactericidal/Fungicidal Concentration of n-butanol Fraction

Test Organisms	**Concentration of n-butanol fraction (mg/ml)**			
	50	**25**	**12.5**	**6.25**
Staphylococcus aureus	-	-	+	+
Escherichia coli	-	-	+	
Candida albicans	-	-	+	

Key: **-** =No growth; + = Growth



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	401.21	1.456	1.114	686.68	393.49	219.244	0.209
2	694.4	43.776	0.074	748.41	686.68	21.998	0.013
3	794.7	43.739	0.309	817.85	748.41	24.751	0.067
4	902.72	41.62	1.066	925.86	817.85	39.92	0.47
5	1111.03	38.356	0.52	1134.18	925.86	83.778	1.662
6	1165.04	38.099	0.292	1195.91	1134.18	25.75	0.087
7	1419.66	37.485	0.122	1435.09	1350.22	35.972	0.042
8	1512.24	38.778	0.476	1535.39	1496.81	15.728	0.095
9	1743.71	36.069	5.753	1982.89	1535.39	180.391	11.411
10	2090.91	43.542	0.449	2168.06	1982.89	66.374	0.392
11	2368.66	42.344	0.32	2384.1	2245.22	50.846	0.171
12	2931.9	33.831	2.531	3024.48	2384.1	271.64	8.004
13	3402.54	33.179	0.138	3425.69	3024.48	188.72	1.977

Figure 1: FTIR Spectrum

Monitoring of the column chromatography of the n-butanol fraction by thin layer chromatography (TLC) revealed component bands that fluoresced under the ultraviolet (UV) lamp set at 365nm and turned brown in the iodine chamber. One fraction (F5) showed a peculiar distinct component band which on re-spotting several times on another TLC plate gave component bands with the same R_f value (0.70). Following this observation, it might be inferred that this fraction contained a single chemical entity or compound. Its dark brown color in the iodine chamber showed that the compound was unsaturated while its exhibition of fluorescence entails that it had one or more of the following characteristics: ultraviolet activity, it contains lone π electrons, it has a cyclic, conjugated structure or it contains a carbonyl structure [12].

This fraction was subjected to spectral analysis (Gas chromatography-mass spectrometry, Fourier transform infrared spectrometry and ultraviolet spectrometry) to enable characterization and identification of its compound ***or*** compounds. The spectra obtained from the Fourier Transform Infrared (FTIR) spectrophotometer (Figure 1) was correlated using the chart of absorption positions of important bonding types [13] as a guide in the absence of computer-based spectral searching system with greater emphasis on the functional group region (above 1500cm^{-1}). It should however be noted that the ranges serve as a guide not an exact assignment.

The following peaks 3402.54cm^{-1} (Intermolecular polymeric O-H stretch or free NH), 2931.90cm^{-1} (C-H stretch), 2090.91cm^{-1} (RC=C-H stretch), 1743.17 (C=O stretch), 1512.24cm^{-1} (C=N stretch for amines and oximes), 1419.66cm^{-1} (O-H deformation for alcohols or phenols) and 902.72cm^{-1} (olefinic C-H out of plane deformation) strongly suggested the presence of these functional groups: O-H (Hydroxyl functional group), C-H, RC=C-H (alkene functional group), C=O (Carbonyl functional group) and C=N**:** (Basic Nitrogen). The suggested O-H functional group has well documented relations to antimicrobial action as observed by Geissman [14] that increased hydroxylation results in increased toxicity. The antimicrobial action of the hydroxyl group is thought to be brought about due to enzyme inhibition by oxidized compounds through reactions with sulfhydryl groups or formation of radicals and non-specific interactions with proteins [15]. The pleasant fragrance of the fraction also supports the presence of the O-H group as alcohols and esters often possess fragrances especially when associated with some degree of unsaturation. The presence of a basic nitrogen atom C=N is indicative that the isolated compound might be an alkaloid.

In conclusion, the leaves of *Pterocarpus santalinoides* have antimicrobial activity and the phytochemical constituents- alkaloids, tannins, saponins, terpenoids, flavanoids and anthraquinones might be responsible for this activity. Fourier Transform Infrared spectral analysis of the isolated component from the n-butanol fraction revealed the presence of O-H, C-H, RC=C-H and C=N functional groups. The antimicrobial effect of the n-butanol fraction can be attributed to the O-H group of its isolated component.

Acknowledgement

The authors wish to acknowledge the support of the Faculty of pharmaceutical Sciences University of Port Harcourt and indeed the entire staff of the Department of Pharmaceutical and Medicinal chemistry in the course of this study.

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