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Antimicrobial, Phytochemical and Cytotoxicity Activities of Extracts of *Lannea welwitschii* (Hiern) Engl. (Annacardiaceae)

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Abstract

Towards discovering new agents to treat microbial infections, methanolic extracts of leaves, stem and roots of Lannea welwitschii were screened for antibacterial and antifungal activity against Staphylococcus aureus (ATCC 25923), Bacillus subtilis (ATCC 6633), Pseudomonas aeruginosa (ATCC 27853), Salmonella typhimurium (ATCC 14028), Proteus vulgaris (ATCC 6380), Escherichia coli (ATCC 25922), Rhizopus spp. (Lab Stock) and Candida albicans (ATCC 18804). Agar cup diffusion method was used to determine zone of inhibition while Minimum Inhibitory Concentration (MIC) on Staph. aureus and E. coli were determined by agar dilution method. Phytochemical screening was done to determine the type of secondary metabolite while cytotoxicity was determined by Brine-Shrimps Lethality (BSL) assay. All the plant parts showed antimicrobial activity against all the test organisms with zone of inhibition of 11.0–20.0 mm, and MIC of 0.31 and 0.62mg/ml on Staph. aureus (ATCC 25923) and E. coli (ATCC 25922) respectively. The LC_{50} from the cytotoxicity test (BSL) were 196.49 and 100.00 µg/mL for the leaves and stem extracts respectively. Saponins, tannins, cardiac glycosides (steroids) and flavonoids were detected. Therefore L. welwitschii as a medicinal plant contains metabolites having broad spectrum antimicrobial activity, and the methanolic extracts were relatively non-toxic.

Introduction

Medicinal plants used to treat infections have produced bioactive constituents that can be used to develop new and effective antimicrobial agents (Newman *et al.*, 2003; R'ios and Recio, 2005). Over 70% of African medicinal plants have not been investigated for pharmacological activities (Cragg *et al.*, 1997; Cowan, 1999), yet the best approach to detect bioactive medicinal plants and compounds is to follow ethnomedicinal uses (Cox and Balick, 1994; Hostettmann, 2000). *Lannea welwitschii* (Hiern) Engl. (Anacardiaceae), a tropical African tree called *ekika* in Yoruba, Nigeria and characterized by the conspicuous "bullet wound" on the bark (Keay, 1989), is used to treat coughs, dysentery, piles and pains (Iwu, 1993; Gill, 1992; Burkill, 1985 and Ayensu, 1978) and is a possible source of antimicrobial agents. Groweiss *et al.* 1997, isolated lanneaquinol and hydroxyl lanneaquinol from the Asian species and the compounds showed morderate antitumour activity against NCI-60 cells. Brine Shrimp Lethality (BSL) assay is a guide to toxicity and anticancer activities of extracts and compounds (Carballo *et al.*, 2002). Olatokunbo *et al.* (2010) reported that aqueous bark extract of *L.*

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welwitschii produced an inhibitory action on gastrointestinal motility and secretion.

This study hereby reports the phytochemical constituents, antibacterial, antifungal and cytotoxicity activities of the leaves, stem and roots of *L. welwitschii.* This is to justify the folkloric uses of the plant in treating infectious diseases locally and establish the cytotoxicity and antimicrobial activities.

Materials and Methods

Plant Materials

The plant parts were collected from the Botanical Garden of University of Ibadan, while identification and authentication of the plant was done at Forestry Research Institute of Nigeria (FRIN) where a voucher specimen with number FHI 107973 was deposited. The leaves, stem and root barks were air-dried, grounded and extracted with cold methanol. The extracts were then concentrated *in vacuo* and stored at 4°C for subsequent uses.

Test Organisms and Media

Bacterial species (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6380 and *Bacillus subtilis* ATCC 6633), and fungi (*Rhizopus* spp and *Candida albicans* ATCC 18804) used were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan. All bacteria were cultured on nutrient agar (No. 2) and nutrient broth (pH 7.4) (Oxoid, England) while fungi species were cultured on Sabouraud Dextrose Agar (SDA) and Tryptone Soy Broth (TSB) (Oxoid, England), and all were maintained on agar slope at 4°C before testing. Brine shrimp *(Artemia salina* Sander®) eggs were purchased from Great Salt Lake Company, USA.

Phytochemical Screening

Preliminary phytochemical screening was carried out according to Evans (2002) and Edeoga *et al.*, (2005). The leaves were tested for the presence of the following secondary metabolites: alkaloids, tannins, anthraquinone glycosides, flavonoids, saponins, steroids and reducing sugars.

Cytotoxicity (Brine-Shrimp Lethality) Assay

Cytotoxicity test was carried out using the methods of Mayer, *et al.,* (1982) and Rahman *et al.*, (2008). Eggs of *Artemia salina* were hatched in sea water for 48-72 h. The nauplii (10 per tube) were put into tubes containing the extracts at three different concentrations (1000, 100 and 10ppm) and the number alive after 24h were used to estimate the number of dead naupli, from where the median Lethal Concentration (LC_{50}) was then computed according to Finney, (1971).

Determination of Antimicrobial Activity

Antimicrobial activity of the extracts was determined by agar cup diffusion method of Perez *et al.*, (1990), with slight modifications (Idowu *et al.* 2006). Extracts were dissolved in methanol to obtain concentrations of 20mg/mL. Each test organism was subcultured in Nutrient Broth, incubated for 24 h to give a 10^{-7} culture (according to 0.5 MacFarland standard) and a 10^{-2} dilution was inoculated into molten but cooled agar. Nutrient agar in seeded plate method was used for bacterial cultures while SDA with surface spread inoculation method was used for fungal cultures. A cork borer of 8.0 mm was used to bore holes into the set agar and the extracts (100 µL) were added to each well. Controls were set up using 10µg/ml gentamicin for bacteria and 1.0 mg/ml tioconazole for fungi, while 40% methanol was used as negative control. The diameter of zones of inhibition was used as a measure of antimicrobial activity. Each test was carried out in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined on *Staph. aureus* and *E. coli* by the agar plate dilution using the method of Andrews (2001). The extracts were mixed with the agar to get a serial dilution of 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15mg/ml in each plate. One milliliter of each dilution of the extract was mixed with 9mL of Mueller Hinton agar, poured into 10cm diameter

Petri dishes and allowed to set. After allowing the agar to dry for about 30min, each plate was inoculated with 1: 100 dilution of overnight broth cultures of each test organisms (containing 1.0 x 10^8cfu/mL according to 0.5 MacFarland standards) and incubated for 24 h at 37°C. Nutrient agar plates with extract but without an organism and one containing only the organism without extract served as positive and negative controls respectively. Each test was carried out in duplicate.

Table 1: Percentage yield of *Lannea welwitschii* extracts

Plant parts	Solvent type	Weight of sample (g)	Weight of extract (g)	Yield (%)
Leaves	Methanol	150.00	14.79	9.86
Bark	Methanol	100.00	11.60	11.60
Stem	Methanol	100.00	6.65	6.65

The plates were then examined for the presence of growth after the incubation period. The lowest concentration of plant extract that showed no visible microbial growth was taken as the MIC.

Results and Discussion

Yields of plant parts to methanolic extractions are 9.86%, 11.60% and 6.65% from leaves, stem and root bark respectively (Table 1). Phytochemcial screening (Table 2) showed the presence of saponins, tannins, flavonoids and cardiac (steroidal) glycosides as the secondary metabolites detected, some of which may be responsible for the observed bioactivity. Tannins, especially has been reported to possess good antimicrobial activity (Scalbert, 1991).

Table 2: Results of Phytochemical Screening of *Lannea welwitschii* Leaf Extract

Secondary metabolites	Tests performed	Colour/ Reaction	Results
Alkaloids	Draggendorff test	No reddish-brown precipitates	-
	Meyer test	No cream precipitates	-
	Wagner test	No reddish-brown precipitates	-
Tannins	Ferric- Cl test	Blue-black colouration	+++
Anthraquinone	Molisch test	No pink colouration	-
Flavonoids	Ammonia tests	Yellow colouration	+++
Steroids	Liebermann-Burchard	Green layer observed	++
	Keller-Killiani test	Brown ring and green layer	++
Reducing sugar	Kedde test	Yellowish-brown colouration	++
Saponins	Frothing test	Frothing observed	++
	Emulsifying test	Stable emulsion formed	++

Key: - negative (not detected), + Present: high ++, higher +++ quantity

Table 3a. Antibacterial activity of leaves, stem and root of *Lannea welwitschii*

Extract	Conc. tested mg/ml	Diameter of zones of inhibition (mm)* on test organisms *Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Proteus vulgaris*	*Escherichia coli*
Leaves	20	13.3±0.6	12.7±0.4	12.1±0.6	11.5±0.2	11.2±0.4	13.0±0.0
	50	15.0±0.0	13.0±0.0	13.0±0.3	12.5±0.4	12.5±0.3	16.0±0.0
Stems	20	18.2±0.2	15.3±0.2	16.1±0.4	14.0±0.0	14.6±0.3	16.4±0.5
	50	20.0±0.3	16.0±0.	18.0±0.0	15.0±0.0	16.2±0.4	17.0±0.6
Roots	20	18.0±0.0	14.8±0.4	16.3±0.6	13.4±0.3	15.2±0.2	12.8±0.3
	50	18.5±0.4	15.0±0.0	18.5±0.3	14.0±0.0	16.0±0.0	13.0±0.3
Genta-micin	10 µg/ml	30.0±0.0	30.0±0.0	28.5±0.2	25.5±0.5	28.0±0.0	28.5±0.5

*Mean of triplicate values ± standard error

Table 3b: Minimum Inhibitory Concentrations (MIC) of Leaf Extracts

Sample Tested	*Staph. aureus* ATCC 25923	*E. coli* ATCC 25922
Leaves	0.31±0.00 mg/ml	0.62±0.00 mg/ml

*Means of duplicate values

Table 4: Antifungal activity of leaves, stem and root of *Lannea welwitschii*

Extracts	Diameter of zones of inhibition (mm) on test organisms	
	Rhizopus spp	*Candida albicans*
Leaves	13.5±0.8	17.4±0.4
Stems	13.6±0.3	16.5±0.6
Roots	12.7±0.2	15.6±0.3
Tioconazole, 0.5mg/ml	20.0±0.0	20.5±0.5

* Means of triplicate values.

All the plant parts showed antimicrobial activity at 20 and 50 mg/ml with extracts of stem and root being more active on the test bacteria (except on *E. coli*) than leaf extract (Table 3a). However the leaf extract showed similar antifungal activity as the stem and root extracts (Table 4). Generally, the antimicrobial activity displayed is broad spectrum in nature being consistently active on Gram positive bacteria (*Staph. aureus* and *B. subtilis*), Gram negative bacteria (*Ps. aeruginosa*, *E. coli*, *S. typhi*, and *P. vulgaris*), mould (*Rhizopus* spp.) and yeast (*Candida albicans*). *Candida albicans* was more susceptible to the plant extracts than *Rhizopus* species. Results of cytotoxicity study (Table 5) showed the LC_{50} as calculated by Finney Probbit Analysis (Computer Software Programme) to be 196.49 and 100.00 µg/ml for the leaf and stem respectively.

In the cytotoxicity study evaluated by the brine shrimp lethality bioassay the LC_{50} values of 196.49 and 100.00 µg/ml for the leaf and stem extracts respectively indicate low cytotoxicity and antitumour property (Pisutthanan, *et al.*, 2004). However the low values for cytotoxicity indicate relative safety for the consumers of the herb. The results are also in agreement with moderate antitumour activity (evaluated by cell line method) reported by Groweiss *et al.* (1997). An allied species, *Lannea coromandelica gave a* LC_{50} of 53.59 while other allied species: *Lannea nigritana* and *Lannea acida* stem barks also showed low and nil cytotoxicity respectively when tested at 500µg/ml (Sowemimo *et a.l.*, 2009).

Conclusion

The research work showed that leaves, stem and root bark of *Lannea welwitschii* possessed secondary metabolites that have broad spectrum antimicrobial activity which may be explored to develop new antimicrobial agents. Further, the folkloric use of the plant to treat infections has been justified while the cytotoxicity status of methanolic extracts has been established. Further studies to isolate and characterize the bioactive constituents are been considered.

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Table 5: Brine Shrimp Lethality (BSL) assay of Extracts of *Lannea welwitschii*

Extracts/ Conc.	1000 ppm		100 ppm		10 ppm		Computed LC_{50} (µg/ml)
	Number of *Artemia salina* alive at 0 min and 24 hrs						
	0 min	24 hrs	0 min	24 hrs	0 min	24 hrs	
Leaf	10	0	10	9	10	10	
	10	0	10	10	10	10	196.49
	10	0	10	9	10	10	
Stem	10	0	10	5	10	10	
	10	0	10	5	10	10	100.00
	10	0	10	5	10	10	

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