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Antimicrobial and antioxidant activity of catechin-3-o-rhamnoside isolated from the stem bark of *Lannea kerstingii* Engl. and K. Krause (Anacardiaceae)

Njinga Ngaitad Stanislaus[1], Sule Mohammed Ibrahim [2], Pateh Umar Usman[2], Hassan Halimatu Sa'adiya[2], Magaji Mohammed Garba[3], Abdullahi Sa'ad Toyin[1], Bakare-Odunola Taibat Moji[1], Ache Roland Ndifor[4], Egharevba Godshelp Osas[5], Shittu Abiodun Oyetunji[6] and Salawu Muritala Salawu[7]

[1]Department of Pharmaceutical and Medicinal Chemistry, University of Ilorin, Ilorin, Nigeria
[2]Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria
[3]Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria
[4]Department of Chemistry, University of Yaoundé I. Cameroon
[5]Department of Chemistry, University of Ilorin, Ilorin, Nigeria
[6]Department of Pharmaceutics and Industrial Pharmacy, University of Ilorin, Ilorin, Nigeria
[7]Department of Pharmacognosy and Drug Development, University of Ilorin, Ilorin, Nigeria

Abstract: Various epidemiological researches have shown that consumption of vegetables and fruits are essential to maintain health and prevent diseases but the emergence of more and more drug resistance bacteria has led to high mortality. Thus the study of the antimicrobial and antioxidant activities of a flavonoid (Catechin-3-o-rhamnoside) isolated for the first time from *Lannea kerstingii.* Catechin-3-o-rhamnoside was isolated using dry vacuum liquid chromatography. It was characterized using ^{1}H-NMR, ^{13}C-NMR and 2D NMR spectra. The antimicrobial activity was determined using agar diffusion and broth dilution method. Antioxidant activity was determined through reaction of the compound with DPPH radical. The compound was active against, *Methicillin Resistant Staphylococcus aureus, S. aureus, B. subtilis, E. coli, K. pneumoniae, S. typhi, S. dysentariae, C. albicans and C. tropicalis* with zone of inhibition ranging from 22.0±0.1 to 35.0±0.2mm and inactive against vancomycin resistant *enterococci*, *Proteus mirabilis* and *C. ulcerans.* The MIC ranged from 6.25 to 12.5µg/ml while the MBC/MFC ranged from 12.5 to 50.0µg/ml. The compound showed a high radical scavenging activity with EC_{50} of 46.87µg/ml. These results show a potential lead drug for resistant bacteria and natural antioxidants.

Keywords: Catechin-3-o-rhamnoside, Lannea kerstingii, antioxidant, antimicrobial.

INTRODUCTION

Infectious diseases are among the main cause of morbidity and mortality worldwide due to drug resistant microorganisms and emergence of unknown disease-causing microbes (Iwu *et al.,* 1999, WHO, 2013). Research has shown that risk of negative clinical consequences, mortality, and high treatment costs with drug-resistant bacteria is generally higher compared to patients infected with the same non-resistant bacteria (WHO, 2015). A report compiled by World health organization (WHO) indicates that if urgent and coordinated action are not taken, the world can be heading towards a post-antibiotic era in which common treatable infections can once again kill (WHO, 2014a).

WHO estimates that majority of the world population (80 %) use herbal medicine for some aspects of their primary health care (Yemoa *et al.,* 2011; WHO, 2014b). Patients as well as health care providers are demanding that health care services be revitalized, with emphasis on person-centred care (Roberti *et al.,* 2012). Treatment of infections with plant-derived compounds is an age-old practice that is globally employed, especially in developing countries (Geyid *et al.,* 2005; De Albuquerque, 2006). Therefore screening of such plants to validate their use in traditional medicine and isolate the active principle(s) is very necessary (Nielsen *et al.,* 2012; Vashist and Jindal, 2012).

Aerobic organisms without adequate supply of oxygen result in hypoxia (Lee *et al.,* 2007). Hypoxia can cause some pathological conditions, such as cardiovascular and cerebral ischemia, inflammation, pulmonary disorders and cancer, as well as in many physiological conditions including high altitude and physical exercise (Miyamoto *et al.,* 2015).

Under hypoxia condition, the production of reactive oxygen species (ROS) increases (Hielscher and Gerecht, 2015). ROS include oxygen radicals (hydroxyl, super oxide, peroxyl and alkoxyl) and certain non-radicals that are either oxidizing agents and/or are easily converted into radicals, such as peroxynitrite, ozone, hydrogen peroxide and singlet oxygen. Reactive nitrogen species (RNS) includes radicals of oxides of nitrogen and products arising when superoxide reacts with nitric oxide

*Corresponding author: e-mail: ngastanjin@yahoo.com

radical, peroxyl and alkoxyl (Wiseman and Halliwell, 1996).

ROS can alter oxidant/prooxidants statues and antioxidant defense system by increasing lipid peroxidation and depleting the antioxidants in cell leading to a condition of oxidative stress (Mansour and Mossa, 2009). ROS can lead to a massive protein oxidation and degradation due to their high chemical reactivity (Nice, 1997; Mimić-Oka *et al.,* 1999).

Pharmacologic strategies targeted at scavenging ROS and RNS may be an effective way of eliminating the physiological damage induced by hypoxia. Therefore, eliminating the damage caused by hypoxia plays a critical role in prophylaxis and therapy. Antioxidant is widely recognized as effective free radical scavenger and can eliminate the ROS by scavenging initiating radicals, binding of metal ions and breaking chain reaction (Khled *et al.,* 2014; Shah *et al.,* 2014).

L. kerstingii Engl. and K. Krause (Anacardiaceae) is widely utilized in traditional medicine. The back is used to treat swellings (Doka and Yagi, 2009), anaemia and malaria (Adoum, 2008; Diallo *et al.,* 2009). The fruits are used against rickets and scurvy (Michel, 2002). Traditional healers in Zaria use the plant to treat hemorrhage, diarrhoea and epilepsy (Njinga *et al.,* 2014). The methanol extract of the stem bark of *L. kerstingii* have been reported to have both antibacterial against *E. faecalis, P. mirabilis, P. aeruginosa, S. aureus, MRSA*, *F. oxysporum* and *S. epidermidis* (Koné *et al.,* 2011).

Though previous studies showed that the plant contains tannins, flavonoids, alkaloids, steroids and triterpenes (Koné *et al.,* 2011; Njinga *et al.,* 2014). There is limited scientific information on the isolation and antimicrobial activity of compounds isolated from this plant in Nigeria. Hence this study is aimed at isolating and determining the antimicrobial activity of flavonoid from the stem bark of this plant.

MATERIALS AND METHOD

General

Thin Layer Chromatography (TLC) were performed using pre-coated plates (MERCK, silica gel 60 F_{254} 0.25mm) at room temperature. Spots were detected by visualizing with an ultraviolet lamp (254 and 366 nm) and then sprayed with 5% sulphuric acid followed by heating at 115 °C for 2 min. Silica gel G for TLC (BDH) and Sephadex™ LH-20 (GE Healthcare) were used as stationary phases for dry vacuum liquid chromatography and column chromatography respectively to fractionate.

Melting points were determined using a melting point apparatus (Gallenkamp). ^{1}H NMR (400 MHz) and ^{13}C NMR (125 MHz) were recorded in DMSO-d6 on a JEOL ECX 400 MHz spectrometer at room temperature. Chemical shifts (δ) are expressed in parts per million (ppm) relative to TMS (δ = 0) as internal standard, while the coupling constants (J values) were given in Hertz (Hz). COSY, HSQC and HMBC were recorded using sine shape gradient pulses.

1,1-Diphenyl-2-picrylhydrazyl (DPPH), vitamin C, were Merck products. All chemicals and solvents used were of analytical grade.

Plant material

The plant was collected in May, 2011 at area BZ, Ahmadu Bello University, Zaria, Kaduna State, Nigeria and identified by a botanist Mal. Umar Galla of the Biological Science Department, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen (1832) was deposited in the herbarium for future references. After identification, the stem bark was dried under shad for two weeks, after which was ground to powder.

Extraction procedure

The powdered stem bark (580g) was defatted (using maceration) with petroleum ether (3x1.5L) at room temperature and shacked occasional for 48 h. The mark was subsequently extracted with ethyl acetate (3 x 1.5L) at room temperature for another 48 h. Both extracts were filtered and concentrated to yield a pale yellow gel petroleum ether extract (4.32g) and a greenish brown solid ethyl acetate extract (10.72g).

Isolation

The ethyl acetate extract (5g) was subjected to dry vacuum liquid chromatography (Pedersen and Rosenbohm, 2001) on silica gel G (Merk) using chloroform ethyl acetate; 25:75 (210ml), 50:50, (90ml), 25:75 (4x90ml), 0:100 (6x90ml) and ethyl acetate: methanol 90:10 (3x90ml) successively. This procedure afforded 15 fractions. Sub-fraction 13 (100mg) was further subjected to continuous gel filtration process using sephadex LH 20. This yielded five main sub-fractions using methanol. The fifth fraction containing the main component was further separated using preparative TLC. The mobile phase was n-hexane chloroform ethyl acetate methanol (3:2:1:1). The band with white fluorescence under UV (R_f=0.3) was scraped and dissolved in methanol. Then the compound was filtered using sintered glass 4 and finally purified using sephadex affording 15mg of compound 1 (catechin-3-O-rhamnoside).

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The scavenging activity of DPPH free radical was determined (Liyana-Pathiana and Shahidi, 2005) as follows. Aliquot of 1.0ml of 0.135mM DPPH prepared in methanol was mixed with 1.0 ml of the compound at different concentrations (31.25, 62.5, 125, 25 and 500 µg/ml) to give final concentrations of 15.625, 31.25, 62.5, 125 and 250µg/ml. The reaction mixture was vortexed

thoroughly and left in dark at room temperature for 30 min. The control solution was prepared by mixing methanol (1.0ml) and DPPH radical solution (1.0ml). The absorbance was measured spectrophotometrically at 517 nm using UV-VIS spectrophotometer (Hitachi, Model 100-20). Vitamin C (50µg/ml) was used as a standard control. The scavenging ability of the compound was calculated using this equation;

DPPH Scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})]\times100$
where $Abs_{control}$ is the absorbance of DPPH + methanol; Abs_{sample} is the absorbance of DPPH radical + sample (i.e. compound).

Tests were carried out in triplicate.

Bacterial and fungal strains
The clinical isolates; Methicillin Resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), *S. aureus, B. subtilis, E. coli, K. pneumonia, S. typhi, S. dysentariae, C. albicans, C. tropicalis, Proteus mirabilis* and *C. ulcerans* were obtained from Ahmadu Bello University Teaching Hospital, Zaria, Kaduna state, Nigeria. The purity of the micro-organisms were checked and maintained in slants of agar.

Assay for antibacterial activity
The test organisms were separately inoculated into tubes of nutrient broth and incubated at 37°C for 18h. The cultures were then adjusted each to 0.5 McFarland turbidity standard and inoculated (0.2ml) onto Mueller Hinton agar (MHA, Oxoid) in petri plates (diameter: 15 cm). A sterile cork borer was then used to make wells of 6 mm diameter on each of the plates containing cultures of the different test organisms. A stock concentration of Compound 1 (50ug/ml) in DMSO was prepared and 0.1 ml was introduced into the wells using sterile Pasteur pipettes. To serve as negative control, 0.1ml of DMSO was introduced in another well. Standard antimicrobials ciprofloxacin and fluconazole (5ug/ml) were included as positive control. The culture plates were allowed to stand for 30 min and were then incubated at 37°C for 24h (bacteria) and 48 h (fungi) respectively. After incubation, the diameter (mm) of the zones of inhibition were measured to determine the activity against the test organisms (Lino and Deogracious, 2006; Njinga *et al*., 2014). The zone of inhibition was measured in triplicates.

Minimum inhibitory/bactericidal concentration
Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was done by broth micro dilution (Tamokou *et al.,* 2009; Fogue *et al.,* 2012) The MIC of the extracts were determined for each of the test organisms at varying concentrations of 50, 25, 12.5 and 6.25µg/ml. These concentrations were obtained by mixing 1ml of the extracts containing double strength of the concentrations (50, 25, 12.5 and 6.25µg/ml) in a test tube with 1ml of nutrient broth. A loop full of the test organism that was previously diluted to 0.5 McFarland turbidity standard was then introduced into the tubes. The procedure was repeated on the test organisms using the standard antibacterial ciprofloxacin and antifungal fluconazole as positive control. To serve as negative control, a tube containing nutrient broth only was seeded with the test organism. All the tubes were then incubated at 37°C for 24 h (bacteria) and 27°C for 48h (fungi) and after, examined for growth by observing for turbidity. The MIC was recorded as the mean of triplicates.

Determination of the minimum microbicidal concentration
This was done by measuring 10 µl aliquot from each well that showed no growth of microorganism from the mixture obtained in the determination of MIC tubes. They were then subcultured on Mueller-Hinton Agar or Sabouraud Dextrose Agar and incubated at 37°C for 24h (bacteria) and 27°C for 48 h (fungi) respectively. After incubation the lowest concentration at which there was no single colony growth of bacteria/fungi was taken as Minimum Bactericidal Concentration (MBC)/ Minimum Fungicidal Concentration (MFC) (Ajaiyeoba, *et al.,* 2003).

STATISTICAL ANALYSIS

The experimental results were presented as mean ± standard deviation (SD). One way analysis of variance was used to perform group comparisons and values at $p \leq 0.05$ were considered to be statistically significant. All computations were done by employing the statistical software (SPSS 16.0; Chicago, IL, USA).

RESULTS

The ethyl acetate extract of the stem bark of *L. kerstingii* was separated by dry VLC on silica gel G to give several sub fractions which were further separated by preparative TLC and purified using cephadex to afford a single compound. The results of the ^{1}H NMR and ^{13}C NMR of the compound 1 are shown in table 1.

Antimicrobial activity of catechin-3-O-rhamnoside
Catechin-3-O-rhamnoside was found to be active against *Methicillin Resistant Staphylococcus aureus (MRSA), S. aureus, B. subtiliS, E. coli, K. pneumoniae, S. typhi, S. dysentariae,C. albicans and C. tropicalis* with zone of inhibition ranging from 22.0±0.1 to 35.0±0.2mm (figure 1). It was inactive against *Vancomycin - resistant enterococci (VRE), P. mirabilis* and *C. ulcerans.* The compound was most active against *K. pneumonia* and *B. subtilis* with zones of inhibition of 35.0±0.2 and 32.0± 0.3 mm respectively.

The MIC of compound 1 against the organisms ranged from 6.25 to 12.5µg/ml (fig. 3). The MIC was 6.25µg/ml for *S. aureus, MRSA, B. subtilis, E. coli, K. pneumonia* and *S. dysentariae* while that for *S. typhi, C. albicans and C. tropicalis was* 12.5µg/ml respectively. The MBC ranges from 12.5µg/ml to 25µg/ml while the MFC was 25 for *C. albicans* and 50µg/ml for *C. tropicalis* (fig. 2).

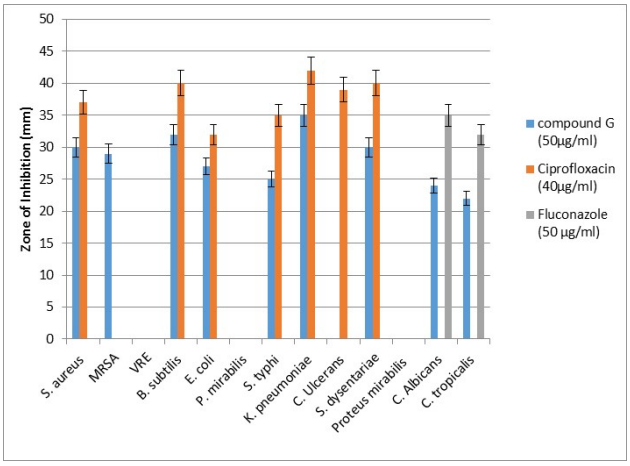

Fig. 1: Zone of inhibition of catechin-3-O-rhamnoside on some disease causing organisms.

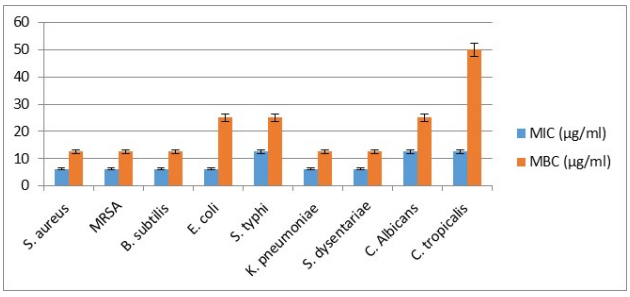

Fig. 2: MIC and MBC/MFC of catechin-3-O-rhamnoside

Compound 1 showed high scavenging activity with increase in concentration (fig. 3). The EC50 was found to be 46.87µg/ml. The EC_{50} is greater than that of ascorbic acid with EC_{50} of 4.72µg/ml.

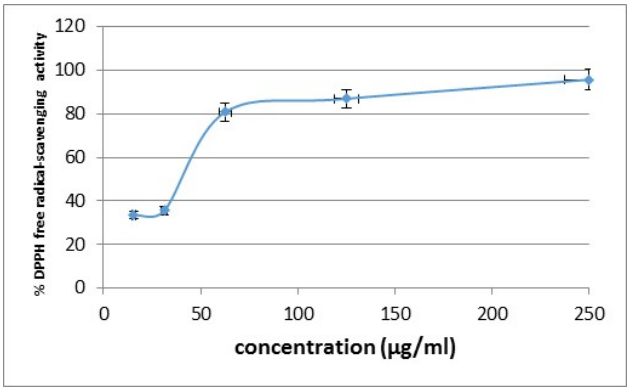

Fig. 3: The % DPPH free radical-scavenging activity of compound 1 at different concentrations.

DISCUSSION

Compound 1 which was gotten from the ethyl acetate extract of the stem bark of *L. kerstingii* after separated by dry VLC on silica gel G, then preparative TLC and purified using cephadex afford a known catechin-3-O-rhamnoside (fig. 4) previous describe in literature (Kim *et al.,* 2012).

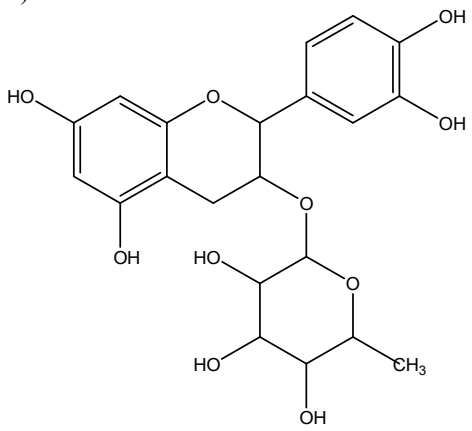

Fig. 4: chemical structure of catechin-3-O-rhamnoside (1): red powdered crystals; $C_{21}H_{24}O_{10}$

The antibacterial and antifungal activities of this compound is greater than those of chloramphenicol (MIC =16-64µg/ml) and nystatin (MIC=128-256µg/ml) (Djouossi *et al.,* 2015), thus showing the good antimicrobial potency of compound 1. Taking into considerations the medical importance of the tested microorganisms, this result is promising in the perspective of developing a new antimicrobial drug. Flavonoids have been shown to have anti-viral, anti-inflammatory, antiulcer, antioxidant, antidiabetic and antimicrobial activities (Kaul *et al.,* 1985; Tencate *et al.,* 1973; Lee *et al.,* 1993; Hang *et al.,* 2002; Vessal *et al.,* 2003). The activity of this compound may probably be due to its ability to complex with bacterial cell walls and to complex with extracellular and soluble proteins.

Flavonoids are also potential antioxidant due to their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Hall and Cuppepett, 1997; Pietta *et al.,* 1998). The antioxidant activity of compound 1 may be due to its ability to scavenge free radicals and active oxygen species.

CONCLUSION

The compound catechin-3-O-rhamnoside isolated for the first time from the stem bark of L. kerstingii possesses significant antimicrobial activity against *Methicillin Resistant Staphylococcus aureus (MRSA), S. aureus, B. subtili S, E. coli, K. pneumoniae, S. typhi, S. dysentariae, C. albicans and C. tropicalis.* The compound also showed

Table 1:[13]C-NMR and [1]H-NMR of catechin-3-O-rhamnoside isolated from the stem bark of *L. kerstingii* compared with that in literature.

Carbon atoms	[13]C-NMR		[1]H-NMR	
	Compound 1	Kim *et al.,* 2012	Compound 1	Kim *et al.,* 2012
2	79	81.4	4.60	4.62
3	73.5	76.1	3.58	3.93
4	28	28.1	2.60/2.65	2.64/2.88
5	156	157.7	-	
6	94.5	96.5	5.70	5.86
7	158	157.0	-	
8	96	96.5	5.90	5.94
9	157	158.1	-	
10	99	100.8	-	
1’	130	132.1	-	
2’	114	115.2	6.73	6.84
3’	146	146.4	-	
4’	146	146.5	-	
5’	114	116.2	6.70	6.77
6’	120	120.0	6.68	6.72
1’’	101	102.3	4.20	4.29
2’’	73	72.1	3.40	3.31
3’’	74	72.4	4.40	3.50
4’’	74	74.1	3.15	2.88
5’’	69	70.5	3.40 m	2.64
6’’	12	18.1	1.18 d	1.25

significant antioxidant activity. Thus this compound can be used as a lead antimicrobial and a potential antioxidant drug.

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