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INHIBITORY EFFECT OF ISOLATED LUPEOL FROM STEM BARK OF *Diospyros mespiliformis* Horsch (Ebenaceae) AGAINST SOME MICROBIAL PATHOGENS

Anas, A*[1]., Ahmed, A[2]., Umar, S[2] ., Jajere, U.M[3] ., Mshelia, E.H[4]. and Natasha, O[4].
[1]Department of Science Laboratory Technology, College of Science and Technology, Hussaini Adamu Federal Polytechnic Kazaure, Jigawa State.
[2]Department of Pharmacognosy and Drug Development, Faculty of pharmaceutical sciences, Ahmadu Bello University Zaria.
[3]Department of Pharmacognosy and Drug Development, Faculty of pharmaceutical sciences, Gombe State University, Gombe.
[4]Department of Pharmacognosy and Ethnopharmacy, Faculty of pharmaceutical sciences, Usmanu Danfodiyo University Sokokto.
[4]Department of Chemistry, University of Pretoria, S/Africa.
Corresponding author: anasringim@yahoo.co.uk

ABSTRACT
Powdered stem bark of plant material (1000g) of Diospyros mespiliformis was extracted with hexane using Soxhlet apparatus and concentrated on a water bath at 60^{0}C. The extract was later subjected to column chromatography techniques using gradient elution in which various fractions were obtained and monitored using thin layer chromatography. The structure of the isolated compound were suggested by 1D and 2D spectroscopic analysis and by direct comparison of the data obtained with those reported in literature to be Lupeol.Antimicrobial screening of the lupeol compound and control drugs were carried out using agar well diffusion method. Significant zones of inhibition of lupeol compound were observed from (30-24mm) and standard control drugs range from (40-32mm). Minimum Inhibitory Concentration(MIC) of the lupeol compound was observed at 6.25-12.5ug/ml and Minimum Bacteriacidal Concentration (MBC) /Minimum Fungicidal Concentration (MFC) ranged from (12.5-25ug/ml).Lupeol compound revealed significant antimicrobial activity against some selected clinical isolates in this study and thus validated the traditional claims ofDiospyros mespiliformis used for the treatment of antimicrobial based infections.
Keywords: Diospyros mespiliformis, Column Chromatography, Lupeol, Inhibitory Effects, Antimicrobial effect.

INTRODUCTION

Diospyros mespiliformis (Ebenaceae) also called "African Ebony" or Kanya by the Hausa of Nigeria is a large deciduous tree confined to tropical and subtropical regions including Africa and Asia (Mohammed *et al*., 2009). Mature trees average 4-6 metres occasionally reaching 5 metres in height. Foliage is dense and dark green with elliptical leaves and fruits often eaten by grazing animals (Belemtougri *et al.,* 2006). The ethno pharmacological uses of different organs of *D. mespiliformis* have been reported in the literature e.g leaf decoctions are used against fever, whooping cough and wounds (Adzu *et al* 2002). Barks and roots are used to treat malaria, pneumonia, syphilis, leprosy, dermatomycoses, diarrhea and facilitation of delivery and as psycho-pharmacological drug (Mohamed *et al.,* 2009). Studies have shown that the leaf extracts of *D. mespiliformis* are effective against *Plasmodium falciparum in vitro* (Etkin, 1997), *Plasmodium berghei invivo* (Adzu and Salawu, 2009) and relieved pains and fever in rodent models (Adzu *et al.,* 2002). Tannins, alkaloids, diosquinone and plumbagin were isolated from *D. mespiliformis* and demonstrated activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* thus providing the basis for the plant usage in herbal medicine *(*Adeniyi *et al.,* 2006; Lajubutu *et al.,* 2006). Root extract of *D. mespiliformis* have shown toxicity potential using sub-chronic administration in mice (Ali *et al*., 2012). Its leaves extract also contained bioactive compounds that exhibits the inhibitory activity against some micro-organisms (Dangoggo *et al*., 2012). The Genus Diospyros such as *D. undulata* has been found to possess the antiproliferative activity against various cancer cells (Rungrojtrakool *et al*., 2012). Another species e.g *D. peregrine* havefound to possess the antitumor and anti-inflammatory activity (Gopal *et al*., 2011 and 2012).

MATERIAL AND METHODS

Collection and authentication of plant material

The plant specimen for the studies was collected from Ringim local government of Jigawa State, Nigeria in January 2013. It was authenticated by a Taxonomist at Biological Sciences Department, Ahmadu Bello University Zaria with voucher specimen number 901431.

Extraction of plant materials
About 1000g of powdered plant material was extracted using Soxhlet apparatus with Hexane 4L for three days (72hr). The content was decanted and then finally filtered with a filter paper (Whatman no. 1). The filtrate was concentrated to dryness using a water bath at 60^0C.

Thin layer Chromatography
A suitable solvent system for Column chromatography was established using Hexane: Ethylacetae (7:3)

Silica- gel Column Chromatography of *Diospyros mespiliformis* Hexane Extract
The hexane extract (3g) was chromatographed over silica-gel 100g (60-120 mesh). Hexane (100%) was used as the initial eluent followed by ethyl acetate gradiently (0 – 10%). Fractions of 30ml each were collected, concentrated and dried under reduced pressure. Column fractions were monitored on Thin Layer Chromatography (TLC) (Merck F_{254}) visualizing under UV (254 nm and 365 nm). P-anisaldehyde reagent or vanillin/sulphuric acid were used as spraying reagent.

Melting point determination for isolated compounds
Small quantity of the isolated compounds was taken in a famed capillary tube and inserted in a column of Stuart (melting point apparatus) the melting point was ascertained from the thermometer when the sample was commenced to melt.

Identification of Compound Isolated
The isolated compound was identified using 1D and 2D NMR (1H,13C,COSY,NOESY and HSQC) and by comparison of data with those reported in the literature. The NMR spectra were obtained at 300 K on ARX-400 MHZ (Brucker/ TOPSPIN).

Evaluation of the antimicrobial activity of the isolated compound
Experimental Design
Collection of clinical isolates
Ten (10) microbial clinical isolates were collected from Department of Medical Microbiology Ahmadu Bello University Teaching Hospital (ABUTH) Shika, Zaria. 0.005mg of the pure compound was weighed and dissolved in 10 ml of $DMSO_4$to obtain a concentration of 50ug/ml. This was the initial concentration of the pure compound used to check the antimicrobial activity of the compound. Mueller Hinton agar was the medium used to grow the microbes. The medium was prepared according to manufacturer's instruction, sterilized at 121^0C for 15 minutes, poured in to sterilized petridishes to cool and solidify. Diffusion method was used for screening of the compound. The sterilized medium was seeded with 0.1 ml of the standard inoculum of the test microbes, the inoculum was spread evenly over the surface of the medium by the use of sterile swab. Using standard cork borer of 6 mm in diameter, a well was cut at the centre of each inoculated medium. 0.1 ml of the solution with concentration of 50ug/ml was then introduced in to the medium. Incubation was made at 39^0C for 24 hrs, after which each medium was observed for the zone of inhibition of growth, and measured with a transparent ruler and the result was recorded in millimeter.

Minimum Inhibitory Concentration
The minimum inhibition concentration of the compound was determined using the broth dilution method. Mueller Hinton broth dilutions were prepared, 10 ml was dispensed in to test tubes sterilized at 121^0C for 15mins, and the broth was allowed to cool. Minimum concentration using Mcfarland's turbidity standard scale number 0.5 was prepared to give turbid solution. Normal saline was prepared with 10 ml was dispensed in to sterile test tube and the test microbe were inoculated and incubated at 37^0C for 6 hrs. Dilution of the test microbe was done in the normal saline until the turbidity marched that of the Mcfarland's scale by visual comparison at this point. The test microbe has a concentration of about $1.5x10^8$ Cfu/ml. Two-fold serial dilution of the compound in the sterilized broth was made to obtain the concentration of 50ug/ml, 25ug/ml, 12.5ug/ml, 6.25ug /ml and 3.125ug/ml. The initial concentration was obtained by dissolving 0.005 mg of the compound in 10 ml of the sterile broth. By obtaining the different concentrations of the compound in the sterile broth, 0.1 ml of the standard test microbe was then inoculated in to the different concentrations of the compound in the sterile broth, incubation was made at 37^0C for 24 hrs, after which each test tube of the broth was observed for turbidity (growth). The lowest concentration of the compound in the sterile broth which shows no turbidity was recorded as the Minimum Inhibition Concentration (MIC).

Minimum Bactericidal Concentration and Minimum Fungicidal Concentration(MBC/MFC)
These was carried out to determine whether the test microbes were killed or only their growth was inhibited. Mueller Hinton agar was prepared, sterilized at 121^0C for 15 mins and poured in to sterile petridishes and the plates were allowed to cool and solidify. The contents of the MIC in the serial dilution were then sub cultured. The prepared medium, incubation was made at 37^0C for 24 hrs, after which the plates were observed for colony growth, the MBC/MFC for the plates with the lowest concentration of the compound without colony growth.

RESULTS AND DISCUSSION
Percentage Yield.
The methanol extracts yielded 0.778w/w.

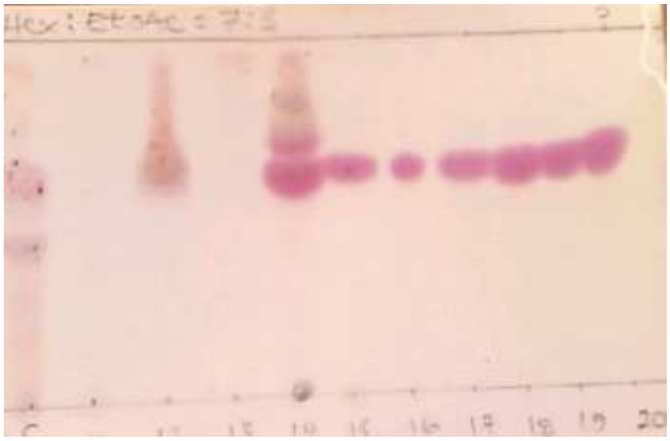

Plate 1. Chromatogram of the fractions 11- 20 coded as DM2 developed in Hex; EtoAc (7:3) sprayed with Anisaldehyde/ H_2SO_4

Melting Point of Compound Isolated

The melting point of compound was found to be 252-253^{0}C .

Table 1: ^{13}CNMR Spectral Data of Isolated Compound and Literature Reported in ppm (in $CDCl_3$)

Carbon	*Lupeol	AA1
1	38.7	38.8
2	27.4	27.4
3	79.0	78.9
4	38.9	38.8
5	55.3	55.2
6	18.3	18.3
7	34.3	34.2
8	40.8	40.8
9	50.4	50.4
10	37.2	37.1
11	20.9	20.9
12	25.1	25.1
13	38.0	38.0
14	42.8	42.8
15	27.4	27.4
16	35.6	35.6
17	43.0	42.9
18	48.3	48.2
19	48.0	47.9
20	151.0	150.9
21	30.0	29.8
22	40.0	39.9
23	28.0	27.9
24	15.4	15.3
25	16.1	16.1
26	16.0	15.9
27	14.5	14.5
28	18.0	18.3
29	109.3	109.3
30	19.3	19.2

* Witchuda *et al* (2007)

Table 2: ^{1}HNMR Spectral Data of Isolated Compound and Literature Reported in ppm (in $CDCl_3$)

H/C Position	*Lupeol	AA1
3	3.17	3.18
4	-	-
5	0.66	0.64
8	-	-
10	-	-
14	-	-
17	-	-
19	2.36	2.02
20	-	-
23	0.94	0.94
24	0.74	0.73
25	0.81	0.80
26	1.01	1.00
27	0.92	0.92
28	0.76	0.76
29a	4.55	4.54
29b	4.67	4.66

*Whitchuda *et al.*, 2010

Table 3: Antimicrobial Activity of Compound

Test organisms	Compound	Ciproflaxacin	Fluconazole
Staphylococus aureus	S	S	R
Staphylococus pygenes	S	S	R
Corynebacterium alcorans	R	S	R
Salmonella typhi	S	S	R
Salmonella paratyphi	R	S	R
Shighella olysenteriae	S	S	R
Escherichia coli	S	S	R
Candida ifrrusei	R	R	S
Candida tropicalis	**S**	R	S
Candida pseudotropicalis	**S**	R	S

KEY: - S= Sensitive R= Resistance

able 4:Zones of Inhibition of the Compound Against the Test Micro-organism

Test organisms	Compound			Ciproflaxacin			Fluconazole		
Staphylococus aureus	2			8	3		7	0	
Staphylococus pygenes		2		4	3		5	0	
Corynebacterium alcorans	0				3		2	0	
Salmonella typhi	2			5	4		0	0	
Salmonella paratyphi	0				3		9	0	
Shighella dysenteriae		3		0		3	8	0	
Escherichia coli	2			7		3	7	0	
Candida ifrrusei	0						0	3	5
Candida tropicalis	2		5				0	3	4
Candida pseudotropicalis	2		4				0	3	2

Table5: Minimum Inhibitory Concentration of Compound Against Test Microbes

Test organism	50 ug/ml	25 ug/ml	12.5 ug/ml	6.25 ug/ml	3.125 ug/ml
Staphylococus aureus	-	-	-	*	+
Staphylococus pygenes	-	-	*	+	++
Corynebacterium alcorans	-	-	0	0	0
Salmonella typhi	-	-	*	+	+ +
Salmonella paratyphi	-	-	0	0	0
Shighella dysenteriae	-	-	-	*	+
Escherichia coli	-	-	-	*	+
Candida ifrrusei	-	-	0	0	0
Candida tropicalis	-	-	*	+	+ +
Candida pseudotropicalis	-	-	*	+	+ +

KEY: - = Clear (No growth), *** = MIC**, + = Turbid (Light growth), **++** = (Moderate turbid), **+++** = (High turbidity) R= Resistance

Table 6: Minimum Bactericidal/Fungicidal Conc. of the Compound against Test Microbes

Test organism	50 ug/ml	25 ug/ml	12.5 ug/ml	6.25 ug/ml	3.125 ug/ml
Staphylococus aureus	-	-	*	+	+
Staphylococus pygenes	-	*	+	++	+++
Corynebacterium alcorans	-		+		
Salmonella typhi	-	-	+	++	+++
Salmonella paratyphi	-				
Shighella dysenteriae	-	-	*	+	++
Escherichia coli	-	-	*	+	++
Candida ifrrusei	-				
Candida tropicalis	-	*	+	++	+++
Candida pseudotropicalis	-	*	+	++	+++

KEY: - = Clear (No growth), * = (MIC/MFC), + = Scanty colonies growth (Light growth), ++ = (Moderate colonies growth), +++ = (Dense colonies growth).

^{1}HNMR of AA_1 : ^{1}HNMR has shown signal at δ 4.5(s) , δ 4.7(s), δ 3.18 (m),δ 2.02(m), δ 0.64(m), δ 0.94(m), δ 0.73(m), δ 0.80(m), δ 1.00(m), δ 0.92(m), δ 0.76(m)ppm.
^{13}CNMR of AA_1: ^{13}CNMR has showed signal at δ150(s) due to quaternary carbon atom, δ78.9(s) is due hydroxyl carbon atom, δ values of δ 38.8, δ 40.8, δ 37.1, δ 42.8, δ 42.9 are due to angular carbon atom which was assigned to C_4, C8, C10, C14, C17.
COSY: Shows H-H correlation at (C-29) at δ 4.66 and H-H (C-3) at δ 3.18 and also correlation between H-H (C-19) at δ 2.02, correlation between H-H at (C-21) at δ value of 1.67ppm, correlation between H-H also occur at (C-11) at δ value of 1.5 and H-H at (C-6) at δ1.3ppm.
NOESY: Spectrum shown correlation between H-29a,H-29b at δ4.54 and δ4.66ppm.Correlation occur at H-3 at δ 3.18ppm.Correlation also occurred on H-19 and H-30 at δ2.02 and δ1.66ppm respectively.
HSQC: Spectrum showed correlation between H-19 and C-19 at δ2.02, H-3 and C-3 at δ78.9, also H-29a and C-29a, H-29b and C-29b at δ4.54 and δ4.66ppm respectively.
The above 1HNMR and 13CNMR spectral were in a comparison of the 1HNMR and 13CNMR signal with those described in the literature of (Whitchuda *et al*., 2010) shown in table 1 and 2 the structure of AA1 to be Lupeol.

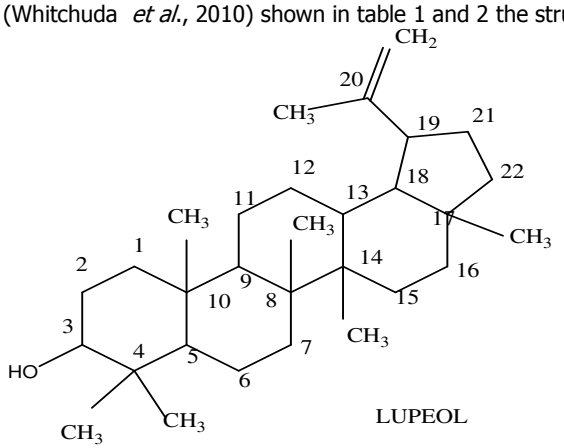

Figure 1: LUPEOL

Table 3. All the clinical isolates screened for sensitivity have shown to be sensitive to the compound (Lupeol) and ciprofloxacin control drug except *corynebacterium alcorans*, *salmonella paratyphi* which have shown resistant to the tested compound (Lupeol) and also*candida ifrrusei* had shown resistant to the pure isolate (lupeol) and this finding was supported by (Jean De Dieu *et al.,* 2011) reported that lupeol was sensitive to *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida tropicalis* and *Cryptococcus neoformans* were the most sensitive to all the tested compounds. Antibacterial activity was expressed as diameter of zones of inhibition (Table: 4). A zone observable inhibition of growth of each micro-organism served as a criterion for declaring acompound sensitive and was indicated by a clear zone around the well. The extent of antimicrobial activity of the compound based on the diameter zones of inhibition has been described as low (12-18 mm), moderate (19-22 mm) and strong activity (23-38 mm) by Ahmad *et al.,* (1999).
Table 4. Indicated the zones of inhibition observed against some clinical isolates by the pure isolated compound compared to control drug (ciprofloxacin and fluconazole). The highest zones of inhibition were observed as 30mm, 28mm, 27mm, 25mm and 24mm against *shigella dysenteriea,staph aureus, E. coli, Salm. typhi and candida tropicalis, candida pseudotropicalis* respectively. The range of zones of inhibition observed in the lupeol compound could be compared with the standard antibacterial and antifungal drug (ciprofloxacin and fluconazole) with the highest zones of inhibition range from 40mm-32mm respectively.

The overall results of zones of inhibition observed in the lupeol compound this study could be compared the classification of zones of inhibition revealed by Ahmad *et al.,* (1999) described as low (12-18 mm), moderate (19-22 mm) and strong activity (23-38 mm) which mostly lupeol compound was concluded to have strong antimicrobial activity in this study. This finding corresponded with the result reported by Halilu *et al.,* (2008) with the same zones of inhibition. Zone of inhibitions of 10mm or more are considered to be significant while zones of inhibition of less than 10mm diameter was considered to be weakly or less active (Faizi *et al.,* 2008).

Conversely, lupeol showed significant zones of inhibition in the cultures of 18 hospital strains of the Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumonia* at a concentration of 30 g/100 L (Ahamed *et al,.* 2007). Zones of inhibition were also observed in *P. aeruginosa, Salmonella typhi* and *Escherichia coli* cultures using lupeol-, betulinic acid- and betulonic acid-impregnated disks at a concentration of 10 mg/mL (Lutta *et al.,* 2008) while lupeol acetate did not display any activity against Gram-negative bacteria and fungi, but displayed a strong antimicrobial effect against Gram-positive bacteria (Freire *et al.,* 2002). The antibacterial activities of lupeol are also conflicting and one of the hypotheses lay in some microorganisms' ability to biotransform the substances yielding different metabolites that possess different activities (Eiznhamer and Xu 2004).

The presence of active compound (Lupeol) may be correlated with the fact that ciprofloxacin showed maximum activity against the bacterial strains compared to isolated lupeol compound which also demonstrated significant antimicrobial activity. The active constituents of plants usually interfere with growth and metabolism of microorganisms in a negative manner (Aboaba *et al.,* 2006). Other compounds like saponins also have antifungalproperties. Many plants release phenolic compounds thatare toxic to microbial pathogens (Aboaba and Efuwape,2001). Hence, these active compounds isolated from the *Diospyros mespelimis* extracts may be responsible for antimicrobial activity of plant extracts which need further investigation.

Table 5. The antimicrobial properties of substances are desirable tools in the control of infections and in food spoilage (Aboaba, *et al.,* 2005). Minimum inhibitory concentration (MIC = 6.25ug/ml) of lupeol compound was observed and higher against gram negative *Staphylococcus aureus, Shigella dysenterieae and E. coli* followed by MIC of 12.5ug/ml observed against *Staph pyogens*, *Candida tropicalis* and *Candida pseudotropicalis* while other clinical isolates resisted because no any sign of turbidity was observed in the trial tubes. The above finding was in lined with report of Jean De Dieu *et al.,* (2011) who observed the MIC of 6.25ug/ml in lupeol, stigmasterol and β-sitosterol against *staph. aureus, candida tropicalis* and *enterococcus faecalis.* In another investigation, lupeol and betulinic acid were inactive against three bacteria species but revealed MICs of 63 and 16ug/ml, respectively, against *Enterococcus faecalis*(TShai *et al.,* 2008).Table 4. Minimum bactericidal concentration (MBC = 12.5ug/ml) of lupeol compound was observed with a higher activity against gram positive *Staph. aureus* follows by *Shigella dysenteries* and *E. coli*(Gram negative)with the same concentration of the compound (12.5ug/ml)while MBC/MFC= 25ug/ml was observed against *Staph. pyogens,C. tropicalis and C. pseudotropicalis* and sign of clear turbidity or resistance was observed in all 50ug/ml tubes concentration (Table 6).Many reports consider the antimicrobial activities of compounds to be significant if the MIC is 10 µg/ml or lower, moderate if 10 · MIC ≤ 100 µg/ml and low if MIC · 100 µg/ml (Kuete, 2010). Referring to these criteria, the tested compounds (Lupeol) had significant to moderate antimicrobial activity with MICs ranged from 12.5 to 25 µg/ml.

MBC and MFC values varied between 25 to 50 µg/ml. Only compound 3 (Lupeol) showed antimicrobial activity against tested microorganisms at different level. This compound was active against *S. aureus* and *P. aeruginosa* (MIC and MBC were 25 µg/ml in both cases), methicillin-resitant *S. aureus* and *E. faecalis* (MIC or MBC were 50 µg/ml in both cases). MBC/MFC of 25 µg/ml against *Bacillus subtilis, Escherichia coli and Salmonella typhi*were reported (Aba *et al.,* 2015). Our results and those obtained by Aba *et al.,* 2015 demonstrated the importance of triterpenes in the antimicrobial activity of *A. ataxacantha*.

In this study, it was observed that gram positive bacteria are the most susceptible to the extracts compared to the gram negative bacteria. These apparent differences in their susceptibility to the extracts might be related to their structural differences in their cell enveloped compositions of gram positive and gram negative bacteria. The gram positive cell wall is simple while that of gram negative is complex consisting of lipoproteins outer membrane and lipopolysaccharides (Jawetze *et al.,* 1978). The outer membrane of gram negative cell envelopes does block the penetration of large molecules and hence the relative resistance of gram negative bacteria to some antimicrobial drugs (Jawetz *et al.,* 1978).

Antibiotic resistance also appears as a result of changes in genes or the acquisition of genes that allow the pathogen to evade the action of antimicrobial drugs. Resistance mechanisms include structural changes in or around the target molecule that inhibit the drug's ability to bind to it; reduced permeability of the cell membrane to the drug and actively pumping the drug out of the cell after it has entered; and production of enzymes that inactivate the antibiotic after it has been taken up by the cell this phenomenon mostly occur in gram negative bacteria (Ken *et al.,* 2012).

CONCLUSION

Chromatographic techniques led to the isolation of pure compound and elucidation and characterization using nuclear magnetic resonance and compared with the data library led to the proposed lupeol structure and this compound revealed significant broad spectrum activity to moderate activity against screened clinical isolates. The antimicrobial activity support and validated the traditional claims used to treat microbial infections.

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