A Phorbol Ester from Antimicrobial and Anthelmintic Leaf Extracts of *Pseudocedrela Kotschyi* Chweinf Harms (Meliaceae)

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ABSTRACT

The powdered dry leaf of *Pseudocedrela kotschyi* was successively extracted with n-hexane, chloroform, 30% aqueous ethanol and methanol. Phytochemical screening of the extracts showed the presence of saponins, tannins, alkaloids, glycosides, cardiac glycosides, flavonoids, phenols and anthraquinones. Antimicrobial screening of the extracts revealed activity against *Staphylococcus aureus, Escherichia coli, Streptococcus pneumonia, Proteus vulgaris, Candida albicans* and *Pseudomonas aeruginosa* with mic values ranging from 100-250 µg/ml. Also, the anthelmintic activity of the extracts against the pin worm, *Entrobius vermicularis* and the earth worm, *Lumbricina terrestris* was investigated and they displayed generally high anthelmintic activity. Chromatographic fractionation ,followed by GC-MS analysis of the fractions led to the identification of phorbol-12,13-dihexanoate as a constituent of the leaves of *P. kotschyi.* This may account for the highly poisonous property of the plant.

Keywords: Pseudocedrela kotschyi, Leaf extracts, Chemical constituents, Phorbol ester, antimicrobial and anthelmintic activities

INTRODUCTION

For centuries, medicinal plants have been used to combat paratism by worms in many parts of the world in ethno-veterinary medicinal practice. For example, extracts of tobacco plant have been used to treat the skin of livestock afflicted with external parasites while the leaves, powdered dry flowers and oil from *Chenopodium ambrosioides*, a shrub from Central America have been used as anthelminthics since the early 1990s (Burkill, 1997). Following folkloric uses, several medicinal plants have been screened for activity using various in-vitro and in-vivo methods and in the process a number of bioactive natural products have been isolated and characterised (Mitscher *et al.*, 1987; Waller *et al.*, 2004).

Helminthosis plays a crucial role in small ruminant production leading to enormous economic losses, particularly in areas where extensive grazing is practised (Perry *et al.*, 2002). *Haemonchus contortus* is a highly pathogenic helminth parasite of small ruminants, which is capable of causing acute disease and high mortality in all age groups, and is one of the top ten constraints of sheep and goat production in East Africa (Musa *et al.*, 2005). Development of resistance to most of the commercially available anthelmintics has become a serious problem worldwide. Also, these drugs are expensive and inadequately available to the resourceful poor farmers of developing countries. Thus, there is need to explore herbal remedies as alternative anthelmintics (Arbonnie,2004). Some plants such as *Lawsonia inermis* L(Lythraceae), *Jatropha*

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curcas L.(Ephorbiaceae) and *Chenopodium ambrosioides* L.(Chenopodiaceae) have been investigated for theirclaimed anthelminthic activity(Oliver,1986; Raoof and Mohamed, 2020) and other biological properties including the promotion of tumour development in animals(Oskoueian *et al*.,2012; Tsai *et al.,* 2016; EFSA CONTAM Panel, 2015; Evans and Farrar, 1998; Veh *et al.,* 2023).

In Nigeria, a plant of ethno-veterinary medicinal use is *Pseudocedrela kotschyi* (Meliaceae). The young stems and roots are commonly used as chewing sticks and in mixtures to treat trypanosomiasis in livestock. The leaves are used in veterinary medicine against instestinal worms (Burkill, 1997).

MATERIALS AND METHODS

Materials

The leaves of *Pseudocedrela kotschyi* were collected from Canza Suleja, Niger State, Nigeria and authenticated at the National Institute for Pharmaceutical Research and Development, Idu-Abuja, and a voucher specimen, No. 6542, was deposited in the herbarium. They were air-dried and powdered using a mortar and pestle.

The solvents used were of analytical grade by BDH Chemicals, Poole, London. The material was stored in a nylon bag and kept in the refrigerator at 4^0C until required. Column chromatography was with silica gel 60-120 mesh manufactured by Burgeoyne Burbidges and Company, Mumbai, India. Thin-layer chromatography (TLC) was run on aluminium sheets 20 x 20 cm coated with silica gel. The spots were visualised using a UV lamp model UVL-21, wavelength 254+ 366 nm, by UVP, Inc., USA.

The antimicrobial screening medium was molten nutrient agar obtained from the University of Abuja Teaching Hospital, Nigeria. The organisms were clinical isolates of *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa* and *Candida albicans,* from the Microbiology Laboratory, University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria.

The proton and 13-carbon NMR spectra were obtained on a Buker AV 300,400MHz, and a Joel 400MHz spectrometers with chloroform as solvent and TMS as internal reference. Peaks were at 7.26 ppm for $CDCl_3$ and 2.50 ppm for DMSO-d6. Gas-chromatography-Mass Spectrometry was carried out on Varian Thermofisher Model of GC-MS, using helium as the carrier gas.

Methods

Extraction and fractionation

The dry powdered leaf (450g) was extracted successively by maceration with 1.5 litres of n-hexane, chloroform and 30% aqueous ethanol, and methanol which on evaporation *in vacuo* gave 3.4g, 6.2g, 17.7g and 3.93 g of residues, respectively.

Phytochemical screening of extracts

The four extracts were subjected to phytochemical analysis using the methods of Sofowora(1982) and Evans(1989) for tannins, saponins, alkaloids, glycosides, cardiac glycosides, anthraquinones, flavonoids and phenols.

Antimicrobial activity screening of leaves extracts of *Pseudocedrela kotschyi*

The antimicrobial activity screening was performed using agar-disc method according to the procedure of Mitscher *et al.* (1987). The zones of inhibition were recorded after 24 hours by measuring the diameter of

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the sensitive (clear) zone around the disc with a transparent ruler calibrated in mm. The absence of any such clear zone means resistance to the plant extrac

Subsequently, the minimum inhibitory concentration (mic) was determined using the tube-dilution technique as described by Cheesbrough (2000). Various concentrations of the extracts were prepared by serial dilutions using distilled water to obtain 500, 450, 350, 250, 150 and 100 µg/ml solutions. The minimum concentration with observed activity was recorded as the mic for each of the extracts or fractions.

Anthelmintic activity screening of the leaves extracts of *Pseudocedracela kotschyi*

The in-vitro method of Eguale *et al*. (2007) was used for the screening. A solution of normal saline was prepared by dissolving sodium chloride pellets (3.4g) in water and made up to 400 ml. A medium was prepared and two species of adult worms, *Enterobius vermicularis* and *Lumbriccina terrestris*, collected from Gwagwalada abbatoir immediately after slaughter were transferred into the medium in petri-dishes. The extracts of the plant were prepared at concentrations of 20,40 and 60 mg/ml and 1ml of each extract solution as well as distilled water as control was introduced into the petri-dish containing seven active(live) worms except for for *Lumbericina terrestris* where 3 worms were used for the control. For each concentration of each extract the number of mobile worms was recorded after 24 hours. In the study, there was no access to a positive control either as a plant extract or a standard drug.

Column chromatographic purification of methanol-soluble extract

The methanol-soluble extract (2g) of the dry powdered leaves of *Pseudocedracela kotschyi* was subjected to column chromatographic purification on silica gel using mixtures of ethanol and dichloromethane. Based on TLC, column collections 35-40% ethanol in dichloromethane and showing similar spots were combined to give fraction ME2. Preparative TLC on silica using cyclohexane/ethanol (8:1) as solvent gave a partially pure fraction, OOG, as a yellow oil (50mg) (R_f value, 0.90).

Gas-chromatography-mass spectrometry (GC-MS) of fraction 00G

The partially pure fraction OOG from the methanol-soluble extract was subjected to gas-chromatography-mass spectrometry analysis to identify any of its constituents.

RESULTS

Extraction and phytochemical screening of extracts

The powdered dry leaf of *Pseudocedrela kotschyi* was successively extracted with n-hexane, chloroform, 30% aqueous ethanol and methanol followed by phytochemical screening of the extracts. The results of the phytochemical screening are shown in Table **1**.

Table **1**: Phytochemical screening results of extracts of *Pseudocedrela koteschyi* leaf

Phytochemicals	Hexane	Chloroform	30% Aqueous ethanol	Methanol
Tannins	–	+	+	+
Saponins	–	–	++	+
Alkaloids	–	–	+	+
Glycosides	–	–	–	+
Cardiac glycosides	+	+	–	–
Anthraquinones	–	–	+	+

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Flavonoids	–	–	++	+
Phenols	–	+	+	+

Key: (++) = very present; (+) = present; (-) = absent

Antimicrobial screening of extracts of *Pseudocedrela kotschyi* leaves

The n-hexane, chloroform, 30% aqueous ethanol and methanol soluble extracts of *Pseudocedrela kotschyi* leaves were screened for antimicrobial activity. The minimum inhibitory concentration values are recorded in Table **2.**

Table **2:** Antimicrobial screening results (mic) of extracts of *Pseudocedrela kotschyi* leaves

Microorganisms	**Extracts/mic(µg/ml)**			
	Hexane	**chloroform**	**30%Aqueous ethanol**	**Methanol**
Sa	100	150	150	250
Ec	150	250	250	250
Sp	250	150	250	150
Pv	150	150	250	150
Pa	250	250	150	250
Ca	150	250	150	250

Key: Sa=*Staphylococcus aureus;*Ec=*Escherichia coli;*Sp=*Streptococcus pneumoniae*; *Proteus vulgaris*; Pa= *Pseudomonas aeruginosa*; Ca=*Candida albicans*

Anthelmintic screening of extracts of *Pseudocedrela kotschyi* leaves

The n-hexane, chloroform and methanol soluble fractions of the powdered dry leaves of P. kotschyi were screened for anthelminthic activity against two species of adult worms, *Enteribius vermicularis and Lumbericana terrestrisin vitro.* The results are shown in Figures **1** a**nd 2,** representing the activity of the extracts at 20, 40 and 60mg/ml for n-hexane, chloroform and methanol solubles per group, respectively.

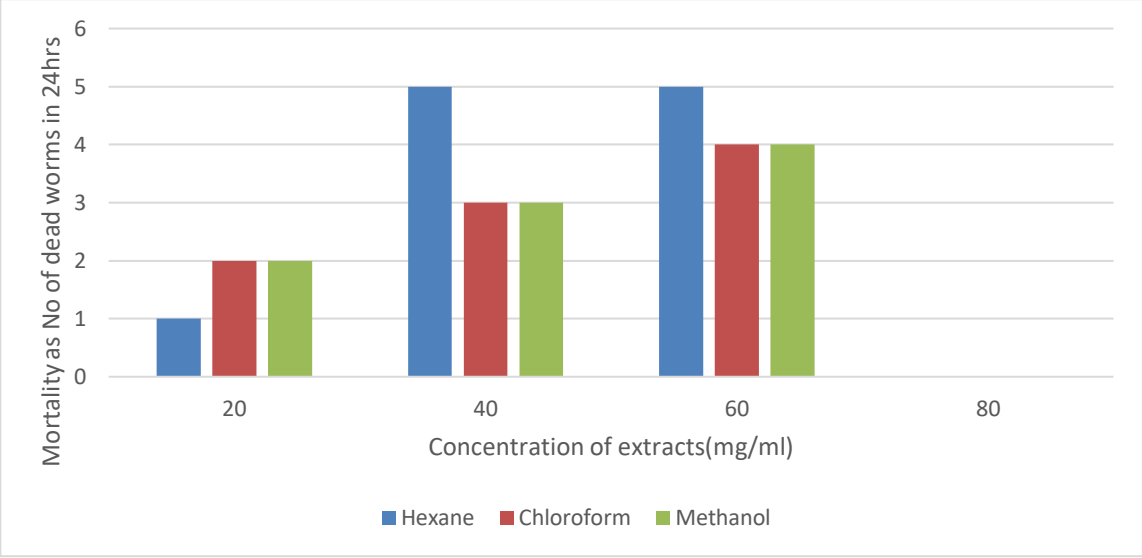

Figure 1: Anthelmintic activity of extracts of *Pseudocedrela kotschyi* leaves against *Enterobius vermicularis* (pin worm)

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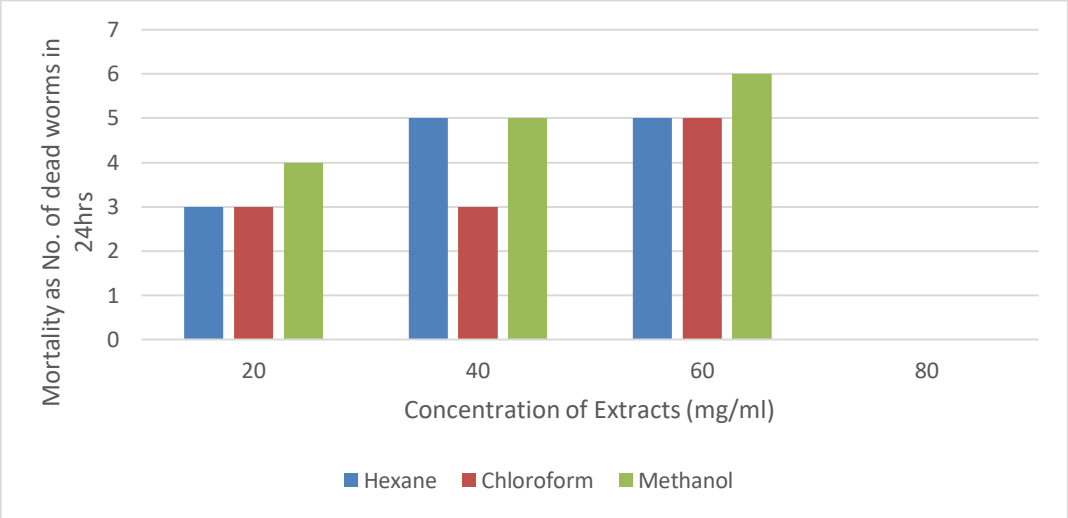

Figure 2**:** Anthelmintic activity of extracts of *Pseudocedrela kotschyi* leaves against *Lumberica terrestris* (earth worm)

Column chromatographic purification of methanol-soluble extract of *Pseudocedrela kotschyi* leaves

The methanol-soluble extract was purified by column chromatography on silica using mixtures of ethanol and dichloromethane (35-40%) and preparative thin-layer chromatography on silica gel to give a partially pure fraction, 00G.

GC-MS analysis of fraction 00G from chromatographic purifications

The partially pure fraction 00G obtained from chromatographic purifications was subjected to GC-MS analysis. The GC of the chromatographic fraction OOG and the MS of component 00G**1** are shown in Figures **3** and **4.**

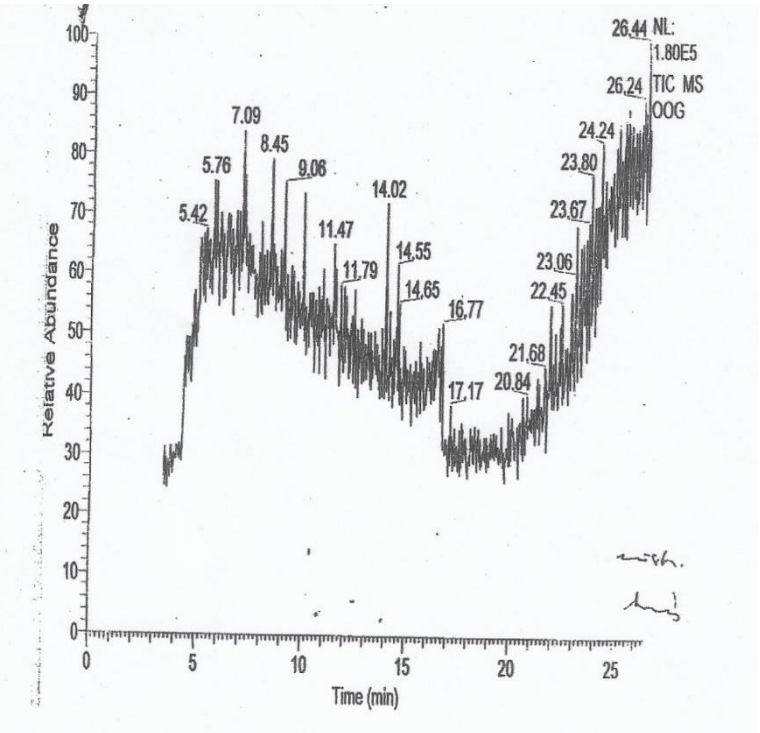

Figure 3: GC of column fraction 00 G of methanol-soluble extract of *Pseudocedrela kotschyi l*eaves

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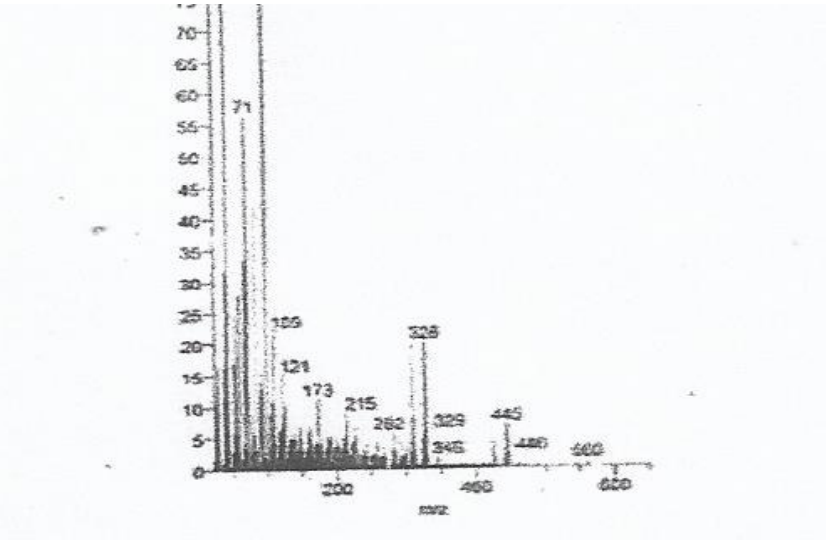

Figure 4: MS of compound from column chromatographic fractionation of methanol-soluble extract of *Pseudocedrela kotschyi* leaves

DISCUSSION

The successive extraction of the powdered dry leaves of *Pseudocedrelakotschyi* gave increasing yields of extracts based on increasing solvent polarity, except that the low yield from methanol after 30% aqueous ethanol was residual and therefore not unexpected.

Phytochemical screening of the extracts showed the presence of tannins, saponins, glycosides, cardiac glycosides, alkaloids, anthraquinones, flavonoids and phenols(Table**1**). These are secondary metabolites that have been established to possess various biological activities, including antimicrobial properties (Esimone *et al.*, 2003; Cowan,1999; Adejumobi *et al.*, 2008).

Among the organisms tested in this work, only 3 have been previously tested against the leaves extracts and the hexane-soluble fraction was found to be inactive against most of the organisms (Satou *et al.,* 2002). However, in this study the four extracts, including n-hexane extract displayed significant antimicrobial activity against all the microorganisms, with mic values in the range 100-500 µg/ml (Table **2**). In fact, the n-hexane fraction showed the greatest activity among the extracts, thus explaining the use of the root and leaves in ethno-medicine to fight various infections (Akande *et al.,* 1998).

From Figures **1** and **2** the in-vitro preliminary anthelmintic screening revealed that *P. kotschyi* leaves extracts possess moderate to high activity against the species of worm tested, *Enterobius vermicularis* and *Lumbericana terrestris.* The results showed that activity increases with increasing concentration. However, the n-hexane-soluble extract was generally more active against *Enterobius vermicularis* than the other extracts, particularly at high concentrations of 40 and 60 mg/l, while the methanol-soluble extract was the most active against *Lambericana terrestris* at the three concentrations, 20,40 and 60 mg/ml.

Also, while the n-hexane was moderately active against the two species of worm, the chloroform-soluble extract was only moderately active against them at 60 mg/ml. The anthelmintic activity may be due to the presence of some phytochemicals, including tannins, terpenoids and flavonoids which have been reported to possess anthelmintic properties (Rarnidra *et al.,* 2008).

Chromatographic purification of the methanol-soluble extract which displayed anthelmintic activity against the two species of worm at relatively low concentrations gave a partially pure yellow oil, OOG, which was subjected to GC-MS analysis. The gas chromatogram (Figure 3) showed a compound OOG1 with a

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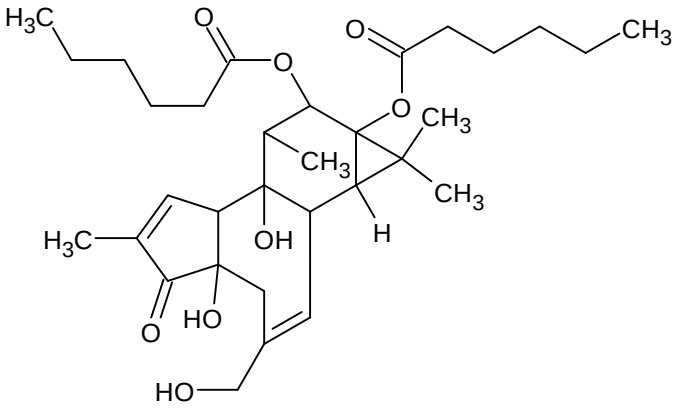
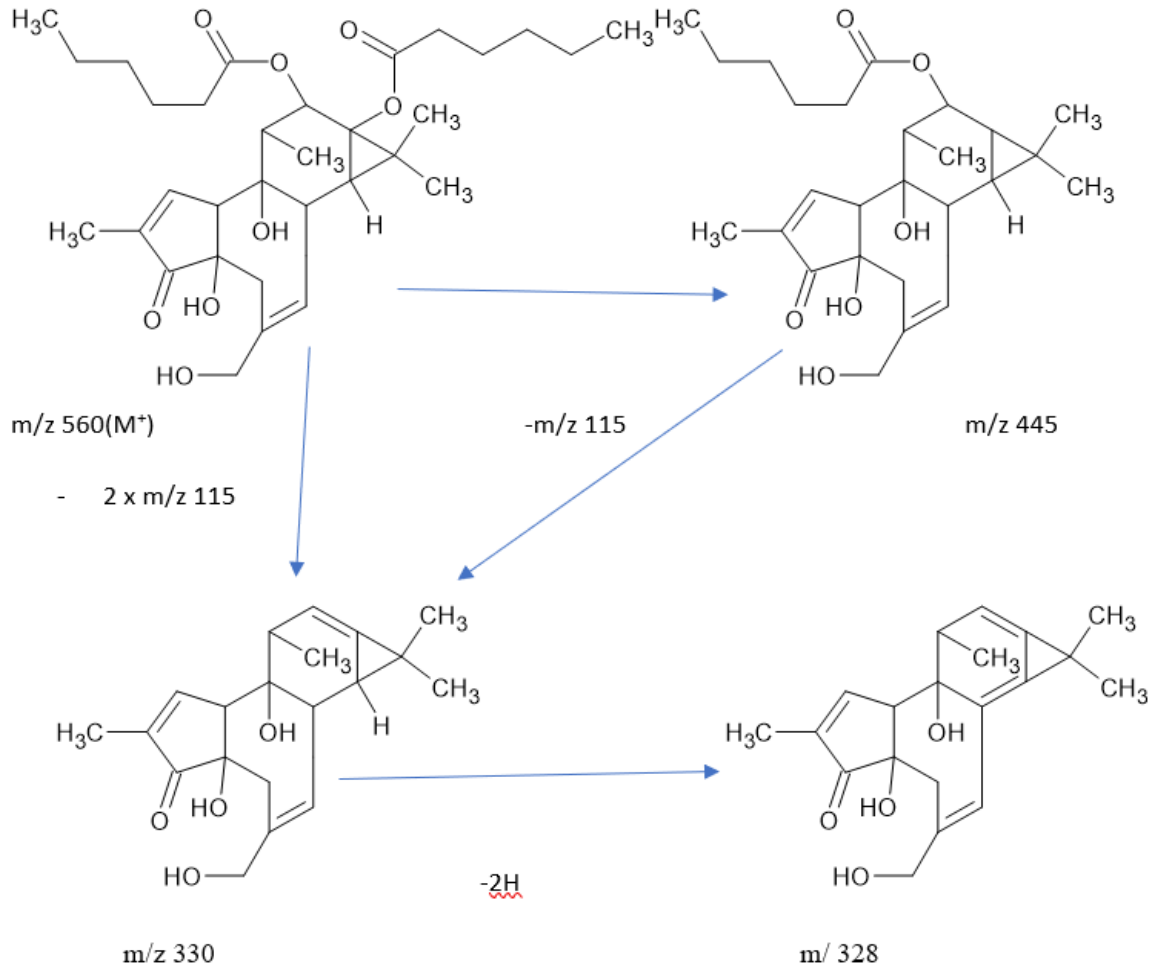
retention time of 8.45 minutes. The MS(Figure 4) showed a molecular ion peak at m/z 560 and fragment mass ion peaks at m/z 445, 330/331, 328, and 113.

Based on the above MS spectral analysis and by comparison with computer library mass spectral data compound OOG 1 has been proposed to be a phorbol ester, probably phorbol-12, 13-dihexylnoate. This was further supported by the presence of the mass ion peak at 228 in the MS which is in good agreement with a previous report (Oragwa *et al.*, 2013) and the proposed mass ion fragmentation pattern shown in Scheme 1**.**



The parent compound, phorbol is a diterpenoid isolated as the hydrolysis of croton oil from Croton tiglium (Flaschentrage *et al.*, 1934).



Scheme 1: MS fragmentation pattern for component OOG 1

The structure of phorbol was determined in 1967(Hecker *et al.*) and its esters are usually unstable and this complicates their MS analysis (Gunjan *et al.,* 2007; Tosa, and Ishizuka, 2017). Phorbol esters possess anti-leukemic activity and are also used as biopesticides and insecticides (Kupchan *et al.,* 1970). They may also be associated with the antimicrobial and anthelmintic activities of the leaves extracts of *Pseudocedrela kotschyi* as their long chain fatty acid hydrolysis products are known to possess antimicrobial and anthelmintic properties(Oragwa *et al.,* 2013).

CONCLUSION

From the results obtained from this work, it can be concluded that the leaves of *Pseudocedrela kotschyi* contain tannins, flavonoids, phenols, saponins, alkaloids, anthraquinones and glycosides. It has also been confirmed that the leaves possess a broad spectrum antimicrobial activity against the tested pathogens with varying degrees of MIC values. From the spectral analysis, it can be deduced that *P. kotschyi* contains the ester, phorbol-12, 13-dihexaonoate, which is biologically active. The antimicrobial activity of *P. kotschyi* leaf extracts therefore may be attributed to this compound. This work records the first isolation of a phorbol derivative from the family Meliaceae and probably accounts for the well known poisonous and anthelmintic property of the leaves of this plant.

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