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ISOLATION OF KAEMPFERIDE AND ANTIMICROBIAL ACTIVITY OF FRACTIONS OF AQUEOUS ETHANOL EXTRACT OF *THESIUM VIRIDE*

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ABSTRACT

Kaempferide was isolated from the ethyl acetate fraction of the aqueous ethanol extract of *Thesium viride* (Santalacea), a plant used in traditional medicine for the treatment of jaundice and ulcer using a combination of silica gel column chromatography and preparative TLC. The structure of this compound was elucidated using NMR spectroscopic analysis and by comparison with reported data. This is the first report of isolation of this compound from this plant. The antimicrobial activity of n-hexane, chloroform, ethyl acetate and n-butanol fractions was determined on some gram negative, gram positive bacteria and fungi. The ethyl acetate fraction was found to have higher inhibitory effect to the test microorganisms.

KEYWORDS: *Kaempferide, Antimicrobial, Thesium viride*

INTRODUCTION

Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Deshmukh et al., 2012). Medicinal plants are used by 80% of the world's population as the only available medicines especially in developing countries. A wide range of medicinal plant part is used for extract as raw drug and they possess varied medicinal properties (Preiyasamy, 2010).

Infectious diseases still represent an important cause of morbidity and mortality among humans, especially in developing countries, resistance of microbes to orthodox drugs have increased and has now become a global concern. This situation forced scientists to search for new antimicrobial substances (Sowjanya et al., 2013). Plant-based antimicrobials represent a vast untapped source of medicines. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu et al., 1999).

Thesium viride Hill (Santalaceae) mainly distributed in Europe, Asia and Africa (Moore et al., 2010). It is a sub-shrub hemiparasite up to 45cm tall, tufted stems starting from a woody rootstock, branched stems, about 2 mm thick, greyish green (Bosch, 2008). It is prescribed to cure ulcers (Polhill, 2005). The aerial part of plant is used in the treatment of jaundice, liver enlargement and splenomegaly (Iwu, 2014). A number of *Thesium* species are employed in African traditional medicine. For example, *T. hystix* roots are used to cure kidney, bladder and lung infections, T. utile is used against gastric disorders and the roots of *T. lacinulatum* are used as a remedy for uterine infections (Belakhdar et al., 2014).

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In this study, we report the isolation and structural elucidation of kaempferide for the first time and evaluation of the antimicrobial activity of the various fractions of aqueous ethanol extract of *Thesium viride.*

MATERIALS AND METHODS

Collection and identification of the plant material

The whole plant of *T. viride* was collected from Karau-karau, Giwa local government, Kaduna State, Nigeria in January 2015. A sample from the freshly collected plant material was identified by the Taxonomist at the herbarium section of the Department of Biological Sciences Ahmadu Bello University, Zaria, where a specimen with voucher number 415 was deposited for future reference. The plant material was then shade dried and ground to a fine powder (1kg).

Preparation of the extract

The powder (1 kg) was macerated in a glass jar with 3.5L of aqueous ethanol (70% v/v) at room temperature for 3 days (72 h). The content of the jar was then filtered through a cotton plug and finally through a filter paper (Whatman no.1), the filtrate was then concentrated to dryness using rotary evaporator to afford aqueous ethanol extract (166 g). Thereafter, 120 g of the extract was suspended in water (700 ml) and then successively partitioned with n-Hexane (3×300 ml), Chloroform (3x300 ml), Ethyl acetate (4×300 ml), and n-butanol (3x300 ml) to afford 0.15 g, 1.21 g, 4.44 g, and 16.26 g weight fractions respectively.

Thin layer chromatography of the various fractions of the extract

Thin layer Chromatography for the various fractions of the extract were developed using different solvent systems and the chromatograms detected using Liebermann Buchard reagent, Borntragers reagent, Dragendoff's reagent, ferric chloride and aluminium chloride

Isolation and purification of the compounds

The ethyl acetate fraction (2 g) was subjected to silica gel column chromatography. The column was eluted with hexane 100%, then hexane-ethyl acetate mixture (9:1, 4:1, 7:3 and 3:2) successively. Base on the TLC profiles, these fractions were pooled together to 3 major fractions (F1 to F3). Repeated silica gel column chromatographic separation of fraction F3 (0.3 g) eluted using hexane (100%), then hexane-ethyl acetate mixture (9:1, 4:1, 7:3) followed by Preparative TLC led to the isolation of a compound coded SAN.

Determination of Zone of Inhibition

The study was designed to determine the susceptibility pattern of various fractions of *T. virides* utilizing a broad spectrum of pathogenic bacteria like gram positive (*Staphylococcus aureus Streptococcus feacalis, Corynebacterium ulcerans*), gram negative (*Escherichia coli, Helicobacter pylori, Salmonella typhii, Shigella dysenteriae*) and fungi (*Candida albicans, C. krusea and C. stellatoidea*). To check the susceptibility pattern of extracts against bacteria and fungi, nutrient agar and potato dextrose agar media inoculated with specific organisms were used. The wells of 6 mm diameter were made equidistantly in the agar plate with sterile borer. The extracts were dissolved in dimethyl sulphoxide and wells were filled with 5 mg/ml of fractions. The plates were incubated for 24 h and 72 h for bacteria and fungi respectively. After incubation the diameters of zone on inhibition were measured.

Determination of Minimum Inhibitory Concentration (MIC) of Fractions

The minimum inhibitory concentration of the various fractions was determined using the broth dilution method. Mueller Hilton broth was prepared; 10 ml was dispensed into test tubes and was sterilized at 121oC for 15 minutes. The broth was allowed to cool. Dilution of the test microorganism was done in the normal saline until the turbidity marched with that of the Mcfarland standard by visual comparison. Two-fold serial dilution of the fractions in the sterile broth was prepared and concentrations were obtained as 5, 2.5, 1.25, 0.625 and 0.313 mg/ml. The sensitive test microbe (0.1 ml) in the normal saline medium was inoculated at different concentrations; incubation was made at 370C for 24hours after which the test tubes was observed for turbidity (growth).

RESULTS AND DISCUSION

The extraction of powdered *T. viride* with aqueous ethanol (70%) for 72 hours gave a yield of 16.67% (w/w) and its successive fractionation with n-hexane, chlorofoam, ethyl acetate, and n-butanol gave a percentage yield of 0.12, 0.99, 3.64 and 13.37% respectively. The results of phytochemical screening of *T. viride* showed the presence of various phytochemicals (Table 1). The fractions n-hexane, chloroform and n-butanol were found to contain steroids and triterpenes. Anthraquinones were found to be present in both chloroform and ethyl acetate fractions. The Yellow fluorescence observed under UV (366nm) after spraying with aluminum chloride from the chromatogram of the ethyl acetate and n-butanol fraction of the extract revealed the presence of flavonoids. Alkaloids were also observed to be present in the n-butanol fraction. This results were based on TLC of the fractions.

Table 1: TLC Phytochemical analysis of *T. viride* Fractions

Compounds	Hexane Fraction	Chloroform Fraction	Ethyl acetate Fraction	Butanol Fraction
Steroids/ Triterpenes	+	+	-	+
Anthraquinones	-	+	+	-
Flavonoids	-	-	+	+
Alkaloids	-	-	-	+

Key: + Present, - Absent

Compound SAN was obtained as amorphous yellow powder soluble in chloroform and methanol. It gave a positive reaction with Ferric chloride and Aluminum chloride under UV-light test suggesting the presence of a flavonoidal nucleus. It showed a melting point of 227-231^{0}C. Its chromatogram (developed using 2:3, hexane/ethyl acetate) gave a yellow spot, an R_f value of 0.66 when sprayed with 20% sulphuric acid.

The ^{1}H NMR spectrum of SAN showed the presence of aromatic protons, two *meta*-coupled doublet at δ 6.18 (H-6) and 6.37 (H-8) and two *ortho*-coupled A_2B_2-type doublet at δ 6.84 (H-3', 5') and 8.01 (H-2', 6') suggested the presence of a *tetra*-substituted and a 1,4-di-substituted phenyl rings. The later ring was further confirmed to be *p*-hydroxyphenyl system from the ^{13}C-chemical shift of the carbon signals at δ 128.8 (C-2', 6') and δ 114.7 (C-3', 5'), which fairly corresponded with those of hydrogen bearing carbons of *p*-cresol (δ 115.3, 130.2). A sharp deshielded signal at δ 12.32 is a result of strong hydrogen bonding between hydroxyl group at C-5 and a carbonyl group at C-4. Two other hydroxyl protons were observed at 9.45 and 10.57 associated with C-3 and C-7 respectively. In aliphatic region the ^{1}H-NMR spectrum displayed an integrated singlet for 3H at δ3.90 which was assigned to -OMe group attached at C-4 position.

The structural of SAN was further substantiated by ^{13}C NMR experiment. Sixteen (16) carbon atoms were identified to make up the compound. A highly de-shielded ^{13}C signal at δc 175.8 ppm indicates the carbonyl carbon at C-4, a benzylic carbon (C-2) at δ 146.6 and oxygen bonded ethylenic carbon (C-3) at δ 135.4. the data above compare well with data from literature is suggestive of the presence of kaempferide. Table 2 gives the proton and carbon-13 data of SAN compared with kaempferide as reported by Lee *et al.,* (2008).

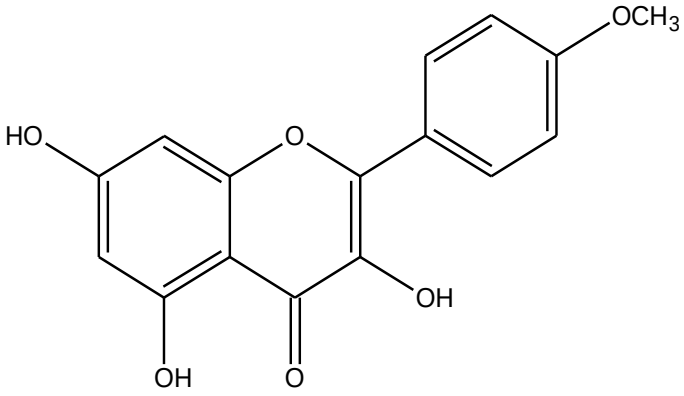

Figure 1: Proposed Chemical Structure of Compound SAN (Kaempferide) Isolated from *T. viride*

Table 2: Comparison between Chemical shift Data of SAN in $CDCl_3$ from *T. viride* and that of kaempferide as obtained from literature

	SAN		Kaempferide [a]	
C	δC (Hz)	δH (Hz)	δC (Hz)	δH (Hz)
2	146.6		146.3	
3	135.4		136.1	
4	175.8		176.1	
5	159.8		160.8	
6	98.1	6.18 d	98.3	6.14 d
7	163.7		164.1	
8	93.2	6.37 d	93.6	6.45 d
9	155.1		156.3	
10	103.0		103.7	
1′	122.2		123.3	
2′	128.8	8.10 d	129.4	8.12 d
3′	114.7	6.84 d	114.1	7.09 d
4′	160.6		160.6	
5′	114.7	6.84 d	114.1	7.09 d
6′	128.8	8.10 d	129.4	8.12 d
3-OH		9.45 s		9.47 s
5-OH		12.32 s		12.43 s
7-OH		10.57 s		10.83 s
4′-OH		-		-
O-CH_3	63.8	3.90 s	55.4	3.83 s

[a] Lee et al 2008

Table 3: Zone of Inhibition of Fractions and control against the test microorganisms (mm)

Test organisms	Hexane	Chlorofoam	Ethyl acetate	Butanol	Ciprofloxacin	Fluconazole
S. aureaus	22	24	27	21	35	-
S. feacalis	21	22	25	20	32	-
C. ulcerans	0	0	0	0	0	-
E. coli	21	25	29	20	37	-
H. pylori	22	26	30	21	30	-
S. typhii	0	0	0	0	41	-
S. dysenteriae	23	24	27	21	39	-
C. albicans	0	0	0	0	-	35
C. krusei	21	22	26	20	-	34
C. stellatoidea	0	0	0	0	-	35

Table 4: Minimum Inhibitory Concentration (MIC) of fractions of *T.viride* against the Test Organisms

Test organisms	Hexane	Chlorofoam	Ethyl acetate	Butanol
S. aureaus	1.25	1.25	0.625	1.25
S. feacalis	1.25	1.25	1.25	1.25
E. coli	1.25	1.25	0.625	1.25
H. pylori	1.25	1.25	0.625	1.25
S. dysenteriae	1.25	1.25	0.625	1.25
C. krusei	1.25	1.25	1.25	1.25

Result of zone of inhibition of bacteria and fungi of the various fractions of the extract of *T. viride* are shown in Tables 3. The result shows some bacteria (*C. ulcerans* and *S. typhii),* and fungi *(C. albicans and C. stellatoidea*) were found to be insensitive to all the fractions. The highest inhibition (diameter of zone of inhibition, 30 mm) was observed in the ethyl acetate fraction against *H. pylori.*

Compared to all other fractions, ethyl acetate fraction showed higher activity against the sensitive bacteria and fungi tested. The antibacterial activity of the extract against the sensitive test organisms greater than 18 mm is term as a very active agent (Ahmed *et al*, 1999).

The Minimum Inhibitory Concentration (MIC) was determined by the broth dilution method and the results were shown in Table 4.

(MIC) is the highest dilution or least concentration of a sample that inhibit the growth of microorganisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. Ethyl acetate fraction was shown to have a lowest MIC of 0.625 mg/ml against all the organisms except for *S. feacalis* and *C. krusei*.

Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with no or lesser side effects due to an array of secondary metabolites (Lee *et al.*1999). Of the four fractions, the ethyl acetate showed the highest activity against both bacterial and fungal test organisms. It may be due to high content of phenolic compounds; anthraquinones and flavonoids which are secondary metabolites that are major group of antimicrobial agents in plant (Cowan 1999).

CONCLUSION

Chromatographic and spectroscopic analysis on the ethyl acetate fraction of the aqueous ethanol extract of *T. viride*, led to the isolation and characterization of kaempferide and The present findings shows of the antimicrobial activity of *T. viride* .The antibacterial activity of ethyl acetate fraction may help the discovery of new classes of antibiotic chemicals that could serve as selective agents against infectious diseases, chemotherapy and control.

COMPETING INTERESTS

The authors declare no competing interests.

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