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RESEARCH ARTICLE

A NEW FLAVAN FROM THE PLANT LEAF EXTRACT OF *CUCURBITA MOSCHATA*

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ABSTRACT

Cucurbita moschata leaves were extracted with methanol to obtain the crude extract. The crude extract was subjected to fractionation using n-hexane, chloroform, ethyl acetate and n-butanol. The phytochemical screening of the fractions obtained revealed presence of flavonoids in the ethyl acetate fraction only. This fraction was subjected to both column and thin layer chromatography which resulted in the isolation of a pure compound. This compound was characterized using both physical and spectroscopic techniques such as UV, IR, ^{1}H NMR and ^{13}C NMR. The isolated compound was identified as 5, 7-diethyl- 3, 4-dimethoxyl-2', 6'-dimethyl flavan.

Key words: *Cucurbita moschata*; Cucubitaceae; Flavonoids, Flavan.

INTRODUCTION

The cucurbitaceae with about 21 genera and 41 species have perhaps more species in cultivation than any other family. Three species of the genius *Cucurbita moschata, Cucurbita maxima, Cucurbita pepo,* occur in Nigeria (Ferriol *et al.,* 2004). *Cucurbita Moschata* is one of the most important vegetable crops in tropical areas. Mature and young fruits, flowers, seeds and young tips of the plant are consumed. *Cucurbita moschata* is rarely found growing in the wild; however it occasionally grows as an escape on dumpsites in most parts of southeastern Nigeria. *Cucurbita moschata* is an annual climbing plant that grows at a fast rate. It flowers from July to September and the seeds ripen from August to October. Full sun is required for successful flowering and fruiting and the plant generally prefer warm temperatures. The fruits are typically ready for harvest from 90 to 120 days after sowing. This annual vine can easily be grown from seed. It is best grown in fertile organically rich medium moisture well drained loamy soil in full sun.

This tendril producing plants can grow quickly to a very large size and are covered with huge lobed green leaves that are coarse hairy and can irritate the skin (Agbagwa *et al.*, 2007). *Cucurbita moschata* is monoecious having both male and female flowers on the same plant. The female flowers are distinguished by the small ovary at the base of the petals. These bright and colorful flowers have extremely short life span and may only open for as short a time as one day. These blossoms are large golden yellow trumpet shaped and pollinated by bees. The plant is self fertile. The male flowers open first on the vine followed by the female flowers distinguished by bulbous ovaries at their bases which develop into mature fruits following pollination. They are used for diverse purposes in different parts of the country ranging from important items in the diets (vegetable) to occupying a special place in the life and culture of many ethnic groups. The leaves as well as the fruits are included in Korean diet. In North America great healing qualities for skin problems such as sores and ulcers are attributed to the seed oil. Traditionally, American Indians use the seeds to treat intestinal infestation and kidney problems and to expel tapeworm. The flowers are used topically to soothe minor injuries. In southern Europe the seeds are also used as an anthelmintic (worm expelling). In India, a paste of the fruit stalks is used to heal boils and earache. Several fatty acids and a steroid glycoside have been isolated and identified by other researchers (Myun *et al*., 2002, Han *et al*., 1999, Chisholm, 1964, Hopkins, 1965). It also contains many vital polyphenolic anti-oxidant flavonoid compounds like α- and β- carotenes, cryptoxanthin-β, Lutein, cucurbitacin and cucurbitane glycosides (Chiu *et al*., 2005). It is rich in B-complex group of vitamins like folates, riboflavin, niacin, vitamin B-6 (pyridoxine), thiamin and pantothenic acid. It also has adequate levels of minerals like iron, zinc, copper, calcium, potassium and phosphorus (Stamp *et al.*, 2005). The main objective of the study was to isolate and characterize flavonoids present in *Cucurbita moschata* and also to determine its antimicrobial activity.

METHODOLOGY

General procedures

The ^{1}H-NMR and ^{13}C-NMR were recorded in deuterated methanol (CD_3OH) on Bruker 300 Ultrashield NMR

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spectrometer measured at 500 and 125 MHz respectively. Chemical shifts are reported in ppm and δ is given in Hz. Melting point was taken on a hot stage Gallen Kamp melting point apparatus with microscope, and was uncorrected. The infrared (IR) was recorded on the Perkin Elmer spectrum FT-IR spectrometer. The ultraviolet (UV) spectra were recorded on Genesis 10 (UV) Scanning spectrometer. Column chromatography was performed on glass column with silica gel 60 230-400 mesh ASTM (Merck 702 TA195034), Thin layer chromatography was carried out on precoated plates with silica gel 60 (Merck 0059381). All solvents were routinely distilled prior to use. Other chemicals were of commercial grade and used without further purification. ACD/ChemSketch application file with file version 3.51.3.51 was used to assist in the structure elucidation.

Plant material

The fresh leaves of *Cucurbita moschata* were collected from Ijanikin area in Lagos State. The plant was identified by a botanist (A. F. Ogundola) of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria. The leaves of *Cucurbita moschata* were dried in the laboratory after collection for a period of two weeks. The air dried leaves sample was pulverized into fine powder. 3.0 Kg of powder sample were stored at room temperature in a clean polyethene bag and well tied to prevent water or moisture from entering the air dried sample.

Extraction and isolation

The air dried and ground plant leaves (3 Kg) of *Cucurbita moschata* was extracted with cold methanol (10L X 6) at room temperature for 24 hours. The combined methanolic extracts were concentrated under reduced pressure. The crude methanolic extract (116.25g) which appeared as dark green powder was suspended in water and sequentially extracted using the solvents n-hexane, chloroform, ethyl acetate and n-butanol. The ethyl acetate fraction was subjected to silica gel column chromatography, eluted with *n*-hex: MeOH with gradual increase in solvent polarity to afford sixty-five fractions. Fractions that show similar pattern on TLC were pooled and combined. Combined Fractions 31-35 were further chromatographed using silica gel column chromatography (*n*-hex: EtOAc: MeOH,) with gradual increasing polarity to yield amorphous brown powder of compound **3** (26.0 mg). The isolated compound was subjected to thin layer chromatography (*n*-hex: EtOAc, 8: 2) to obtain 24.2 mg of the pure compound **3**. Combination of fractions 40-43 yielded quercetin (compound **2**) (14.0 mg) while combination of fractions 46-48 gave kaempferol (compound **1**) (16.0 mg). The isolated known compounds were subjected to thin layer chromatography using the solvent system ethyl acetate: chloroform in the ratio 1: 9 and 9: 1 respectively.

Antimicrobial screening

Organisms

Clinical isolates *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Salmonella typii, Proteus vulgaris and Aspergillus niger* were collected from Baptist Medical Center, Ogbomoso, Nigeria.

Preparation of the medium

2.8 g of nutrient agar was prepared by dissolving in 100 mL of distilled water. The medium was sterilized in an autoclave at 121^{o}C for 15 minutes. It was cooled to 45^{o}C and poured into sterile Petri dishes to solidify.

Preparation of test samples

1.0 mg of the isolated flavan was dissolved in 1.0 mL of dimethylsulphoxide (DMSO). The activity of Streptomycin, contrimoxazole, erythromycin and gentomycin standard antibiotics were also determined and used as the positive control while DMSO was used as the solvent.

Disc diffusion test

Disc diffusion method by Bauer *et al*., (1966) was employed. Filter paper discs were used as carrier for the antibacterial agents. The sterilized discs cut from Whatman no. 1 filter paper were impregnated with solutions of the antibacterial agents. The solvent was evaporated and the disc dried properly. The impregnated discs were placed on the surface of the nutrient agar which has been inoculated with the test organism. The antimicrobial agent upon contact with the agar diffused in all directions. The ability of the test organism to grow or not in the presence of the test sample was then determined within 24 hours by measuring the zones of inhibition. The incubation of the plates was carried out at 37^{o}C. All tests were done in quadruplicate and the antimicrobial activity expressed as a mean of inhibition diameters (mm) produced by the isolated compound with contrimoxazole, erythromycin and gentamycin as the standard antibiotics.

RESULTS AND DISCUSSION

Spectral data of the isolates

Compound 1: Kaempferol

Yellow needles (MeOH); mp 275-277 ^{o}C; UV λ_{max} (MeOH) nm 266, 290 (sh),320 (sh), 367; IR ν_{max} (KBr): 3314 (OH), 1661 (C=O), 1613, 1569,1508 (Ar), 1225, 1009 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD): δ 12.10 (1H, s, 5-OH), 9.76, 9.50, 9.09 (each 1H, s, -OH), 8.15 (2H, d, *J* = 9.0Hz, H-2', 6'), 7.01 (2H, d, *J* = 9.0Hz, H-3', 5'), 6.35 (1H, d, *J* = 1.8Hz, H-8), 6.26 (1H, d, *J* = 1.8 Hz, H-6); ^{13}C NMR (125 MHz, CD_3OD): 145.6 (C-2), 135.5 (C-3),175.3 (C-4), 161.5 (C-5), 98.6 (C-6), 163.3 (C-7), 93.6 (C-8), 156.9 (C-9), 102.7 (C-10), 122.8 (C-1'),132.5 (C-2'), 116.1 (C-3'), 161.7 (C-4'), 116.1 (C-5'), 132.5 (C-6').

Compound 2: Quercetin

Yellow powder; mp 310-312 ^{o}C; UV λ_{max} (MeOH) nm (log ε): 254 (4.26), 368 (4.12); IR (KBr) ν_{max}: 3462, 3375, 3292 (OH), 1672 (C=O),1614, 1549, 1522 (Ar), 1213, 1095 cm^{-1}; ^{1}H NMR(500 MHz, CD_3OD): δ 6.18 (1H, d, *J* = 2.0 Hz, H-6), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 6.88 (1H, d, *J* = 8.4Hz, H-5'), 7.63 (1H, dd, *J* = 8.4, 2.0 Hz, H-6'), 7.73 (1H, d, *J* = 2.0 Hz, H-2'); ^{13}C NMR (125 MHz, CD_3OD):δ 145.8 (C-2), 135.6 (C-3), 175.5 (C-4), 161.4 (C-5), 98.5 (C-6), 163.8

(C-7), 93.7(C-8), 156.4 (C-9),102.9 (C-10), 121.9 (C-1'), 115.1 (C-2'), 145.0 (C-3'), 147.5 (C-4'), 115.5 (C-5'), 120.2 (C-6').

Compound 3: 5, 7- diethyl-3, 4 –dimethoxy-2', 6' -dimethyl flavan,

Brown powder, wt: 24.2 mg, mp: 148-150°C, molecular formula $C_{23}H_{30}O_8$, UV λ_{max} (MeOH) nm: 220, 247, 331; IR (cm^{-1}): 3400 (OH), 3068, 1604, 1464 (Ar), 1380 and 1163; ^{1}H NMR (500 MHz, CD_3OD): δ 8.90 (5H, OH), 5.45 (1H, d, *J* = 5.12 Hz, H-2), 4. 58 (1H, d, *J* = 1.02,H-4), 3.76 (1H, d, *J* = 5.10 Hz), 3.50 (3H, s, H-3a), 2.87 (2H, q, *J* = 0.21, H-5a), 2.43 (3H, s, H- 8a), 2.20 (9H,s, H-8a, H-2', H-6'), 2.65 (2H, t, *J* = 0.21Hz, H-5a, H-7a), 1.25 (6H, t, *J* = 7.56Hz, H-5b, H-7b); ^{13}C NMR (125 MHz, CD_3OD): δ 147.12 (C-3', C-5'), 136.85 (C-8),135.78 (C-6), 132.98 (C-8a), 130.65 (C-4'), 118.65 (C-1'), 115.89 (C-4a), 112.74 (C-5, C-7, C-2'), 109.21 (C-6'), 85.98 (C-3), 74.87 (C-2), 71.95 (C-4), 59.55 (C-3a, C-4b), 22.54 (C-5a), 17.78 (C-5b, C-7b), 15.14 (C-7a) 13.32 (C-2a', C-6a).

The methanolic leaf extract of *Cucurbita moschata* was concentrated to obtain a dark greenish residue. The residue was suspended in distilled water and sequentially partitioned between n-hexane, chloroform, ethyl acetate and n-butanol. Phytochemical analysis of these fractions revealed presence of flavonoids in the ethyl acetate fraction only. Repeated chromatography of the ethyl acetate soluble layer on silica gel gave two known compounds kaempferol (compound 1) and quercetin (compound 2) which were identified by their R_f values 0.78 in the solvent ratio ethyl acetate: chloroform (9:1, v/v) and melting point (275-277°C) and 0.33 in the solvent system ethyl acetate: chloroform (1:9, v/v) (Sutthanut *et al*., 2007; Tewtrakul *et al*., 2009) and their spectroscopic data; and a new flavan (compound 3) which is a derivative of flavan-3, 4-diol. It was isolated as a brown powder with melting point of 148-150°C. The UV data of the isolated compound showed absorbance at wavelength of 331, 247 and 220 nm which is within the range of a characteristic absorption band of a typical flavan skeleton. The IR spectrum

Table 1: Antimicrobial activity of the isolated flavan

Microorganisms used	Zones of inhibition (mm)
Pseudomonas aeruginosa	inactive
Staphylococcus aureus	inactive
Escherichia coli	inactive
Salmonella typii	inactive
Aspergillus niger	inactive

Table 2: ^{1}H NMR data (in ppm) for isolated compound (DMSO, 300MHz)

Carbon position	^{1}H (δ)		Protons
2	5.45	(1H, J = 4.98)	-C-H
3	3.76	(1H, J = 4.95)	-C-H
4	4.58	(1H, J = 4.95)	-OC-H
3a, 4b	3.50	(3H)	$-OCH_3$
5a	2.65	(2H, J = 0.21, 7.54)	$-CH_2$
5b	2.43	(3H, J = 7.42)	$-CH_3$
7a	2.87	(2H, J =7.54, 0.21)	$-CH_2$
7b	1.25	(3H, J = 7.42)	$-CH_3$
2a', 6a'	2.20	(6H)	$-CH_3$
	8.90	(5H)	-ArOH

Table 3: ^{13}C NMR data (in ppm) for isolated compound (DMSO, 125MHz)

Carbon position	^{13}C (δ)	Carbon type
2	74.87	Cyclic (CH)
3	85.98	Cyclic (CH)
4	71.95	Cyclic (CH)
5, 7	112.74	Aromatic (C)
6	136.85	Aromatic (C)
8	135.78	Aromatic (C)
1'	118.65	Aromatic (C)
2', 6'	109.21	Aromatic (C)
4'	13.65	Cyclic (CH)
3', 5'	147.12	Aromatic (C)
3a,4b	59.55	Methoxy (OCH_3)
4a	115.89	Aromatic (C)
5a	22.54	Alkyl (CH_2)
5b, 7b	17.78	Alkyl (CH_3)
7a	15.14	Alkyl (CH_2)
8a	132.98	Aromatic (C)
6'a, 2'a	13.32	Alkyl (CH_3)

indicated the presence of hydroxyl group ($3400cm^{-1}$), alkyl group ($2758cm^{-1}$), aromatic (1601 cm^{-1}, $1464cm^{-1}$) and C-O ($1163cm^{-1}$) absorption bands. The ^{1}H and ^{13}C NMR spectra (Tables 1, 2 and 3) revealed two set signals, which features indicated a flavonoid with a flavan skeleton.The ^{1}H NMR spectrum of the compound gave signals indicating the presence of aromatic hydroxyl, two methylene and two aromatic methyl protons, at δ 8. 9, 2.65, and 2.20 respectively, two methoxy protons at δ 3.5, methyl alkyls at δ 1.25 and methine protons at δ 3.84, 4.38 and 5.45. The first indication of the position C-3 as substitution site as shown in Figure 1. was evidenced from the ^{1}H NMR spectrum, which did not show the typical signal assigned to H-3. These data were further supported by ^{13}C NMR spectrum displaying a total of 23 carbon atoms. Aromatic carbons showed signals as between δ_C 147.8 and δ_C 117.3, methoxyl carbons occurred at δ 59.55, methylene carbon signals were observed at δ22.54 and 15.14 while methyl carbon signals were observed at δ17.75 respectively.

The isolated flavan did not exhibit any inhibitory effect on the following microorganisms, *Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Escherichia coli, Salmonella typii and Aspergillus niger* as shown in Table 1 Mori *et al*., (1987) and Tsuchiya *et al*., (1996) observed a relationship between the structures of the flavonoids and their activity against *Proteus vulgaris* and *Staphylococcus aureus*. Most of the activity was observed to be related to the presence of hydroxyl groups at positions 3', 4', 5', 6' in ring B and at C-3. Despite the presence of hydroxyl groups at positions 3', 4', 5' in ring B the isolated compound was inactive. This inactivity can be attributed to the absence of hydroxyl groups at positions 3, 5 and 7. The C-2, C-3 double bond which is absent was also noted not to be crucial for antibacterial activity (Zheng *et al*., 1996). Stapleton *et al*., (2004) noted that substitution at the 6 or 8 position with a long chain aliphatic group enhance activity. The isolated flavan has alkyl substitution at positions 5 and 7. It was also noted by Alcraz *et al*., (2000) that the presence of methoxy groups drastically reduces the antibacterial activity of flavonoids. The presence of two methoxy groups at positions 3 and 4 could have nullified any activity that the substitutions at positions 3', 4', 5' in ring B might have produced.

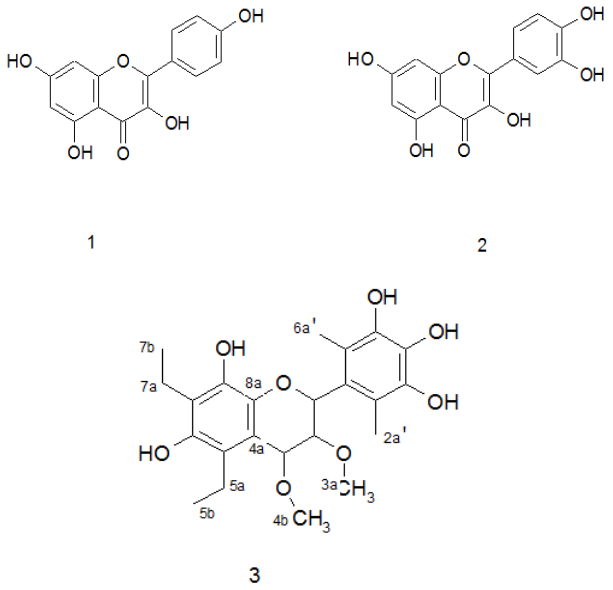

Figure 1. Chemical structure of isolates

Conclusion

The phytochemical study of *Cucurbita moschata* has led to the isolation of two known compounds (kaempferol and quercetin) and a new flavan. The suspected isolated flavan was identified as 5, 7- diethyl -3, 4-dimethoxy- 2', 6'- dimethyl flavan. The structure of the suspected compound was established based on the data obtained from the UV/Vis, IR, ^{1}H NMR and ^{13}C NMR as shown in Figure 1. ACD/ChemSketch application file (file version 3.51.3.51) was used to aid in the elucidation of the structure of the isolated compound.

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