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ANTIFUNGAL ACETYLATED FLAVONOL FROM THE SUDANESE MATERIAL OF VANGUERIA MADAGASCARIENSIS RUBIACEAE

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ABSTRACT

The authors report on the isolation of a flavonol from the Sudanese material of *Vangueria madagascariensis*. The flavonoid was isolated from the ethanolicextract by column and thin layer chromatography. The structure was elucidated by a combination of analytical tools (UV, IR, ^{1}H NMR, MS). In cup plate agar diffusion assay, compound I and the chloroform fraction of *Vangueria infausta* were evaluated for their antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*). The chloroform fraction did not show antibacterial activity, but it showed significant inhibitory activity against the fungi: *Candida albicans* and *Aspergantillus niger*. Compound I also showed antifungal activity. However, it did not reveal antibacterial activity.

KEYWORDS: *Vangueria madagascariensis*, Isolation, Flavonol, Antimicrobial Activity,

INTRODUCTION

Flavonoids are among the most ubiquitous group of plant secondary metabolites distributed in varios plants. During the past decade an interesting number of publications on the health benefiting effects of flavonoids, such as those on cancer and coronary heart diseases have appeared

Flavonoids encompass a large group of polyphenolic substances which are widely distributed in plants and foods of plant origin. Flavonoids share a common basic skeleton consisting of two benzene rings joined by a linear three carbon bridge. They are divided into subclasses: flavans, flavones, flavonols, flavanones, isoflavones, aurones, chalcones, and anthocyanins.[1-4] Recently, and due to their health benefits, research on the of flavonoids gained an increased pulse.

In vitro, flavonoids are effective scavengers of free radicals[5,6] Several prospective cohort studies have examined the relationship between some measure of dietary flavonoid intake and coronary heart disease (CHD) risk[7-14] Some of these studies have found that higher flavonoid intakes is associated with significant reduction in CHD risk[7-11,15] In general, the foods that contributed most to total flavonoid intake in these cohorts were black tea, apples, and onions.

Different *In vitro* and *in vivo* studies revealed that some flavonoids exhibit antimicrobial potential[16-23] others exert anispasmodic activity.[24] Several flavonoids have been shown to have potential as hepaoprotective agents[25] Flavonoids like gossypin and morin were found to show significant analgesic activity.[26]

Vangueria is a genus of flowering plants in the family Rubiaceae. The genus contains over 50 species distributed in Africa south of the Sahara with one species occurring in Madagascar (*V. madagascariensis*). The centre of diversity is in east Africa (Kenya, Tanzania) and they are rare in west Africa.[27]

Several *Vangueria* species - *V. latifolia*, *V. pygmaea*, *V. thamnus* - are known to cause heart failure four to eight weeks after ingestion of certain rubiaceous plants.[28]

Vangueria infausta, the medlar or African medlar, is a species of plant in the family Rubiaceae, which is native to the southern and eastern Afrotropics. The fruits are consumed by humans and have a pleasant apple- like flavor. The specific name *infausta* alludes to the misfortune believed to result from its use as firewood.[29,30]

MATERIALS AND METHODS

Materials

Plant material

The fruits of *Vangueria madagascariensis* were collected from Niala, west Sudan. The plant was identified and authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum.

Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV-Visible Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer.Mass spectra were run on a Joel Mass Spectrometer (JMS-AX500).

Methods

Extraction of Flavonoids from the fruits of *Vangueria madagascariensis*

Powdered shade -dried fruits (1kg) of *Vangueria madagascariensis* were extracted with 95% ethanol at ambient temperature for 24h. The solvent was removed under reduced pressure giving a brown solid.

Column chromatography

Open column (80x 4 cm) was used for fractionation of the ethanolic extract. Silica gel with particle size 100-200 mesh from LOBA chemicals was used as stationary phase. The column was packed with slurry of silica gel with chloroform and then allowed to equilibrate for two hours before use.

The column was successively eluted with chloroform: methanol (4:1), (3:1) and (2:3).The ratio 3:1(chorofrom: methanol) gave a fraction rich in flavonoids and it was further purified by TLC.

Preparative thin – layer chromatography

(0.5g) of the crude product was dissolved in minimum amount of 95%ethanol and applied on (20x20 cm) silica gel plates as narrow strips. The plates were developed with (chlorlform: methanol; 3: 2) and the chromatograms were located under UV light .Bands were scratched and eluted from silica with absolute ethanol .After filtration, the solvent was removed *in vacuo* to leave compound I .

Stepwise procedure for the use of shift reagents

-Methanolic solution of the compound was first recorded in UV.
-3 drops of NaOMe were added to the cuvette and after mixing, the NaOMe spectrum was recorded.
-6 drops of $AlCl_3$ reagent were added to the methanolic solution of the flavonoid, and the $AlCl_3$ spectrum was measured.
-3 drops of HCl were then added and after mixing, the $AlCl_3$/HCl spectrum was measured.
-Powdered NaOAc was added to fresh flavonoid stock solution in the cuvette, the mixture was shaked and the NaOAc spectrum was recorded.
-NaOAc/H_3BO_3 spectrum was then measured after adding H_3BO_3

Antimicrobial assay

In cup plate agar diffusion assay, compound I and the chloroform fraction of *Vangueria madagascariensis* were evaluated for their antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*).

Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^o C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 10^8. 10^4 colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0-02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature, and then incubated at 37^o C for 24 hours.

Preparation of fungal suspensions

Fungal cultures were maintained on Saboraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed with sterile normal saline and the suspension was stored in the refrigerator until used.

Testing for antibacterial activity

The cup plate agar diffusion method was adopted to assess the antibacterial activity .(2 ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^o C in water bath.(20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle. In each of these plates, which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed a test solution.

The agar discs were removed and cups were filled with (0.1 ml) of each test solution and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37^o C for one hour. After incubation the diameters of the resultant growth inhibition zones were measured.

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar Saboraud dextrose

agar was used. Samples were used here by the same concentrations used above.

RESULTS AND DISCUSSION

Characterization of isolated compound

A successive silica gel column chromatography followed by further purification via thin layer chromatography allowed for the isolation of a flavonoid – compound I. Identification of this compound was based on extensive UV shifting reagents,IR, ^{1}HNMR and mass spectroscopy data.

Compound I was isolated from fruits of *Vangueria madagascariensis* as yellow solid.The IR spectrum (Fig.1) displayed absorption bands at ν(KBr) 673(C-H , Ar.) ,1105 (C-O) ,1550 , 1645 (C=C , Ar) 1689 (C = O) , 2854 (C- H) aliphatic ,3429cm^{-1} (OH).

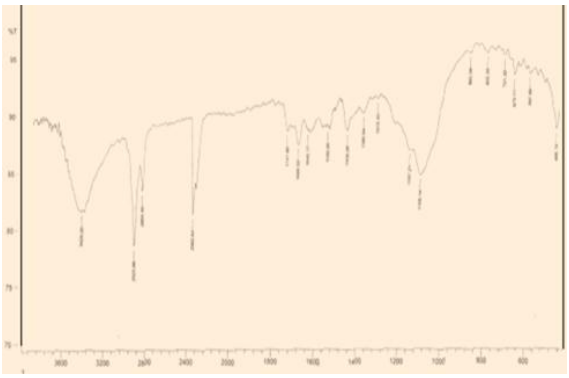


Fig. 1: IR spectrum of compound I.

The U V spectrum (Fig.2) showed λ_{max} (MeOH) 273 nm. The appearance of one band indicates that the compound is probably a dihydroflavonol, dihydrochalcone, flavanone or an isoflavone. Among these classes only dihydroflavonols are characterized by a 3-OH function. The sodium methoxide spectrum indicated the presence of a 3-OH function (Fig.3).

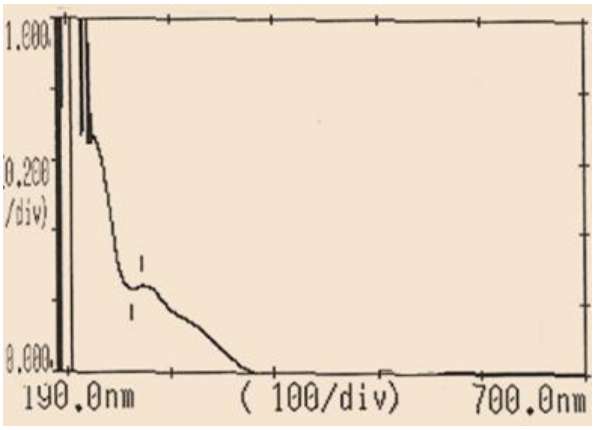


Fig. 2: UV spectrum of compound I.

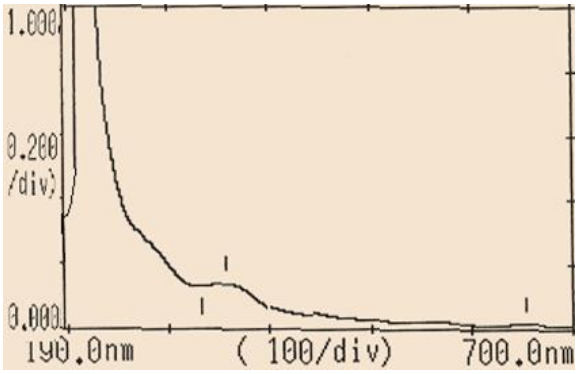


Fig. 3: Sodium methoxide spectrum of compound I.

The shift reagent sodium methoxide is a strong base capable of ionizing all hydroxyl functions in the flavonoid nucleus , but it is diagnostic of 3- and 4'- OH groups .In both case it gives a bathohromic shift but with decrease in intensity in case 3-OH. Addition of the strong base NaOMe to a methanolic solution of compound I caused (Fig.3) a bathochromic shift (84nm) with decrease in intensity. This indicates the presence of a hydroxyl group at C-3.

To investigate the hydroxylation pattern of this isolate , the UV shift reagents were employed .Sodium acetate is a weaker base than sodium methoxide and as such ionizes only the more acidic hydroxyl group in flavonoids i.e. 3-,7- and 4-' hydroxyl groups. Since ionization of 7- hydroxyl group mainly affects band II, sodium acetate is particularly useful diagnostic reagent for the specific detection of 7-hydroxy function.

However, no bathochromic shift was observed in the sodium acetate spectrum (Fig.4).This indicates absence of a 7-OH function.

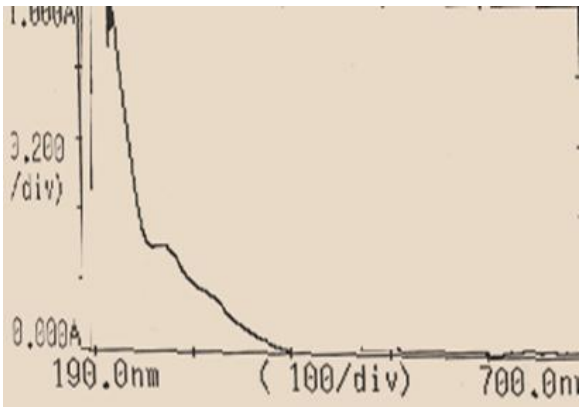


Fig. 4: Sodium acetate spectrum of compound I.

Aluminum chloride chelates with functional groups such as the 5-hydroxy-4-keto-, 3-hydroxy-4-keto and ortho dihydroxyl systems and this is evidenced by bathochromic shifts of one or both bands in the spectrum .

$AlCl_3$ complex between the C-4 keto function and either 3- or 5.- hydroxyl group is stable in presence of HCl acid . However catechols yield acid-labile complexes.

The aluminium chloride spectrum did not reveal any bathochromic shift (Fig.5) indicating absence of 3- and 5-OH function as well as catechol systems.

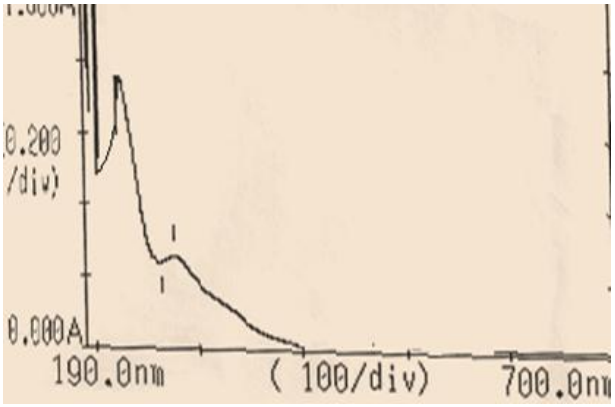


Fig. 5: Aluminium chloride spectrum of compound I.

The ^{1}HNMR spectrum (Fig. 6) showed signals for one methyl group at ∂1.23(s,3H) and three acetyl functions at ∂ 1.89 (s,9H). The resonances at ∂6.10 (s, 1H) and ∂6.70 were assigned to H-6 and H-8 respectively.The C-8 proton usually resonates at lower field relative to C_6-H due to the deshielding influence of neighboring oxygen at position 1. The B-ring protons appeared as singlets at ∂ 6, 98 and ∂7.31ppm.

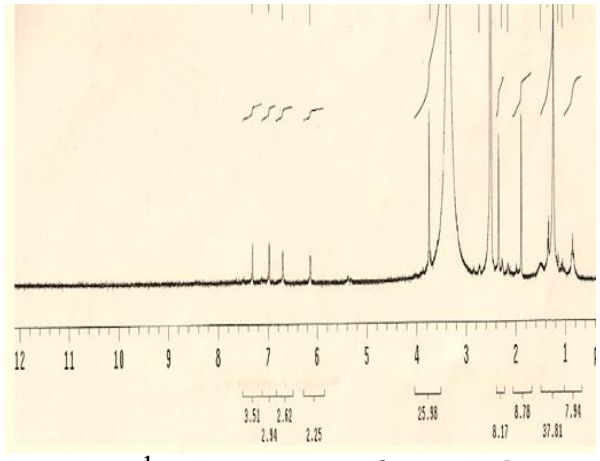


Fig. 6: ^{1}H NMR spectrum of compound I.

The ESI-MS (Fig.7) showed a molecular ion peak at m/z 381 [M^++H] .Other important fragmentns resulting from retro Diels-Alder fission(Scheme I) were shown at m/z 134, 246.Apparantly these correspond to intact A and B rings respectively.

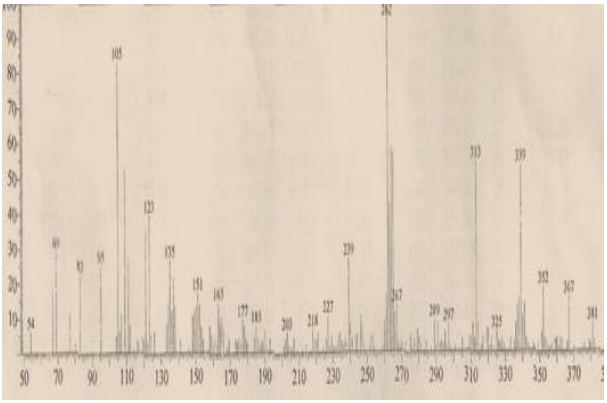
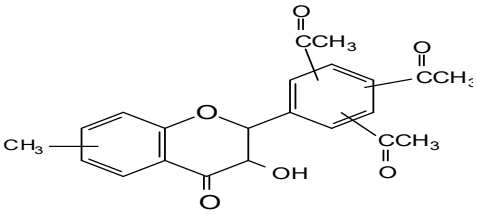
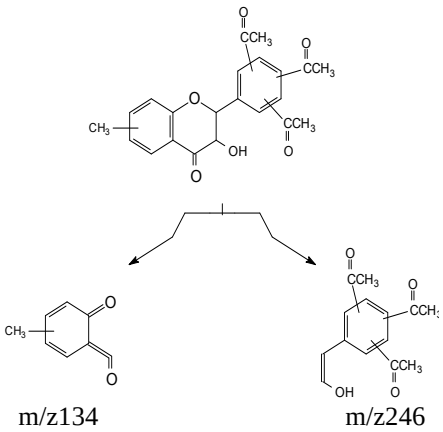


Fig.7: Mass spectrum of compound I.

On the basis of the above cumulative data, the following tentative structure was proposed for compound I.



Compound I



m/z134 m/z246

Scheme I: Retro Diels-Alder fission of compound I

Antimicrobial activity

In cup plate agar diffusion bioassay, compound I and the chloroform fraction of *Vangueria madagascariensis* were evaluated for their antimicrobial activity.

The chloroform fraction did not show antibacterial activity, but it showed significant inhibitory activity against the fungi: *Candida albicans* and *Aspergantillus niger*. Compound I also showed antifungal activity. However, it did not reveal antibacterial activity (Table 1). A tabulation of the antimicrobial activity of standard chemotherapeutic agents is shown in tables 2 and 3.The activity is expressed as less active, if the zone of inhibition is 9-12 mm, moderate: 13-15mm; very active if the inihibition zone is greater than 18mm.

Table 1: Antimicrobial activity of compound I.

Sample	Conc. mg/ml	E. c	P. s	B. s	S. a	C. a	A. n
Comp. I	100	-	-	-	-	17	19
$CHCl_3$ Extract	100	-	-	-	-	14	15

Table 2: Antibacterial activity of standard chemotherapeutic agents.

Drug	Conc. mg/ml	B.s	S.a	E.c	P.a	S.t
Ampicillin	40	15	30	-	-	-
	20	14	25	-	-	-
	10	11	15	-	-	-
Gentamycin	40	25	19	22	21	22
	20	22	18	18	15	17
	10	17	14	15	12	14

Table 3: Antifungal activity of standard chemotherapeutic agent.

Drug	Conc. mg/ml	A.n	C.a
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

- S.a*: Staphylococcus aureus*
- E.c*: Escherichia coli*
- P.a: *Pseudomonas aeruginosa*
- A.n: *Aspergillus niger*
- C.a: *Candida albicans*
- *B.a: Bacillus subtilis*

M.D.I.Z: Mean diameter or growth inhibition zone (mm). Average or two replicates.

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