Isolation and Characterization of a Flavonone From the Leaves of Sudanese *Geigeria alata*(Benth and Hook)- Asteraceae

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Abstract

Phytochemical screening of *Geigeria alata* leaves rev*ea*led the presence of flavonoids,tannins and alkaloids. A flavonoid - compound I- was isolated from ethanol extract by column and thin layer chromatography and its structure was established on the basis of its spectral data (IR, UV, NMR and MS). Compound I and different fractions (ethanol, chloroform, n- butanol and ethyl acetate) of *Geigeria alata* were screened for their antimicrobial activity against six standard human pathogens. The ethyl acetate fraction showed excellent antibacterial activity against all test organisms.However, the ethanol extract was inactive against the test bacteria ,but it gave good antifungal activity against *Aspergillus niger* The chloroform and n-butanol fractions exhibited moderate activity against *Escherichia coli* .

***Keywords*:** Isolation**,** Characterization**,** Flavanone, *Geigeria alata*, Antibacterial Activity

I-Introduction

Some natural products- like plant phenolics- tend to modulate human metabolism in a manner beneficial for the prevention of a wide range of diseases[1]. Plant phenolics are important plant secondary metabolites [2] . They primarily act as antioxidants and reflect various beneficial effects in a multitude of human disorders[3, 4].

Flavonoids are plant phenolics characterized by low molecular weight[7-9] . Flavonoids play a vital role in photosynthesizing cells[10]. The original "flavonoid" research started with the work of the Hungarian scientist Albert Szent-Gyorgi back in 1936[11]. Flavonoids are characterized by flavan nucleus[8] and a C_6-C_3-C_6 carbon skeleton[12, 13]. They constitute a group of structurally related secondary metabolites with a chromane-type skeleton which may have a phenyl substituent in C_2- or C_3 position[14]. The basic structural feature of flavonoids is : 2-phenyl-benzo-γ- pyrane nucleus consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C)

[10]. Different substituents(hudroxyl, methyl, acetyl,isoprenyl …etc) may occur in the flavonoid nucleus and according to their molecular structure, flavonoids are divided into eight major groups[13,15]: flavones , flavonones, flavonols , isoflavones , anthocyanidins , catechins , dihydroflavonols and chalcones. In plants, flavonoids are often present as O- or C-glycosides[14].

Geigeria is a genus comprising about 30 species[16] in the Asteraceae family. *Geigeria alata* (Benth. And Hook)-locally known as "Gad-gad" is among the key species used by local healers in western Sudan. It is claimed to treat diabetes, cough, intestinal disorders and epilepsy. The plant has many pharmacological effects. In some *in vitro* studies ,the ethanol extract of *Geigeria alata* exhibited a cytotoxic effect against some tumor cell lines. Also a cytotoxic sesquiterpene lactone was reported from this species[17-19]. *Geigeria alata* essential oil showed moderate *in vitro* cytotoxicity and exhibited weak anti-HIV activity[20].

In continuation of our interest in the phytoconstituents of plants used in Sudanese system of medicine , this study was designed to investigate the flavonoids of *Geigeria alata* which is widely used in Sudanese ethnomedicine to treat a wide spectrum of diseases.

2-Materials and Methods

2.1-Materials

2.1.1-Plant material

Leaves of *Geigeria alata* were collected from Kordofan- west Sudan.The plant was authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum.

2.1.2-Instruments

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

2.1.3-Test organisms

The following standard microorganisms were used to assess the antimicrobial potency of compound I and different fractions of *Geigeria alata* : *Bacillus subtilis* (Gram +ve) , *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve) , *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans* and *Aspergillus niger* .

2.2-Methods

2.2.1-Preparation of plant extract for phytochemical screening

(100 g) Of powdered air- dried leaves of *Geigeria alata* were extracted with 95% aqueous ethanol (soxhlet) until exhaustion. This prepared extract (PE) was used for phytochemical screening.Phytochemical screening was accomplished according to the method described by Harborne[21].

2.2.2-Extraction of flavonoids

(1 kg) of powdered air-dried leaves of *Geigeria alata* was macerated with 95% ethanol (5L) for 48hr at room temperature with occasional shaking and then filtered off . The extraction process was repeated two more times with the same solvent. Combined filtrates were concentrated under reduced pressure at 40^{o} C yielding a crude product.

2.2.3-Thin layer chromatography

Analytical (TLC) was carried out using aluminium sheets precoated with kiesel gel 60F 254 of 0.2 mm thickness. The aim was to detect a suitable solvent system for separation of flavonoids; to monitor fractions from column and to determine the purity of isolates.

The spotted (TLC) sheets were developed in the saturated vapour chromatographic tanks by using suitable solvent systems. The spots were then viewed in UV light using both (λ_{max} 366 nm and λ_{max} 254 nm) with and without exposure to NH_3 vapour.

2.2.4-Column chromatograpy

Open column (80× 4 cm) was used for fractionation of the ethanol extract of *Geigeria alata*. Silica gel with particle size 100-200 mesh from LOBA chemicals (India) was used as stationary phase.

The composition of the mobile phase (chloroform: methanol) was determined by TLC analysis.The column was packed with slurry of silica gel with chloroform and then allowed to equilibrate for two hours before use.

The ethanolic extract *Geigeria alata* was fractionated by a silica gel column eluted with chloroform: methanol (4:1). (10ml) Fractions were collected. Fractions F7- F32 were pooled together on the basis of their TLC pattern. The concentrated fractions were further purified by

TLC using silica gel developed by chloform: methanol (5:1; v: v).The chromatograms were viewed under UV light and the chromatogram with Rf 0.70 was eluted from silica with absolute ethanol to give compound I. The purity was checked by TLC experiments using silica gel and the solvent systems: (i) ethyl acetate saturated with water (ii) BAW (5:1:6) and finally (iii) methanol: toluene (2:1).

2.2.5-Biological activity

2.2.5.1-Antimicrobial assay

Compound I and different fractions of *Geigeria alata* were assessed for antimicrobial activity against six standard pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans and Aspergillus niger*) . The cup plate agar diffusion bioassay was used with some minor modifications.

2.2.5.2-Preparation of bacterial suspensions

Aliquots(1ml) of 24 hours broth culture of the test organisms were distributed into agar slopes and incubated at 37^{o} C for 24 hours. Bacterial growth was harvested and washed off with sterile normal saline, and then suspended in 100 ml of normal saline to give about 10^{8}- 10^{4} colony forming units per ml. The Average number of viable organism per ml was determined using of the surface viable counting technique.

Serial dilutions of the stock suspension were prepared in sterile normal saline. (0.02 ml) of the appropriate dilution was transferred into the surface of dried nutrient agar plates. After drying, the plates were incubated at 37^{o} C for 24 hours.

Fungal cultures were maintained on Sabouraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed off with sterile normal saline and the suspension was stored at 4^{o}C until used.

2.2.5.3-Testing for antimicrobial 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 a water bath. (20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle and each plate was 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 for a test solution.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 at 37 ^{o} C for one hour.After incubation the diameters of the resultant growth inhibition zones were measures in duplicates and averaged.

For antifungal activity, instead of nutrient agar Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above.

3-Results and Discussion

3.1-Characterization of compound I

Phytochemical screening of the leaves of *Geigeria alata* revealed the presence of tannins, flavonoids and alkaloids. From the ethanol extract compound I was isolated by column and thin layer chromatography and its structure was elucidated via a combination of spectral techniques (UV, IR, ^{1}HNMR and MS).

The IR spectrum of compound I (Fig.1) revealed ν (KBr): 699,754,777,861(C-H,aliph., bending),1093(C-O),1448(C=C, Ar.),1604(C=O) , 2941(C-H,aliph.) and 3380cm^{-1}(OH).

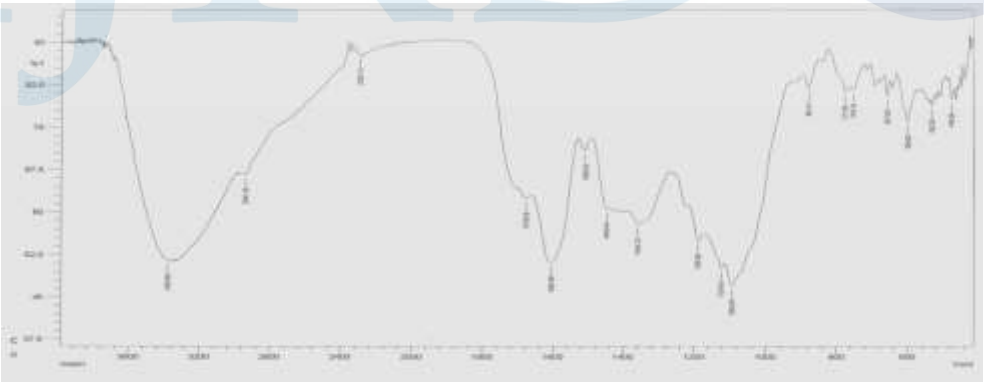


Fig.1: IR spectrum of compound I

In the UV, compound I absorbs at λ_{max}257nm (Fig.2). Such absorption is characteristic of : flavanones, dihydroflavonols and isoflavones. However, no shoulder in the region 300-340nm (which is characteristic of isoflavones) was observed.

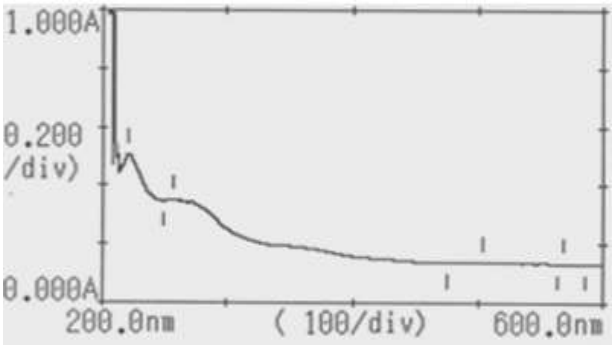


Fig.2: UV spectrum of compound I

The sodium methoxide spectrum (Fig.3) did not reveal a bathochromic shift diagnostic of a 3-OH function which is acharacteristic feature of dihydroflavonols.However, it revealed a bathochromic shift characteristic of a 4` -OH function. Such findings suggest that the isolated compound is a flavanone.

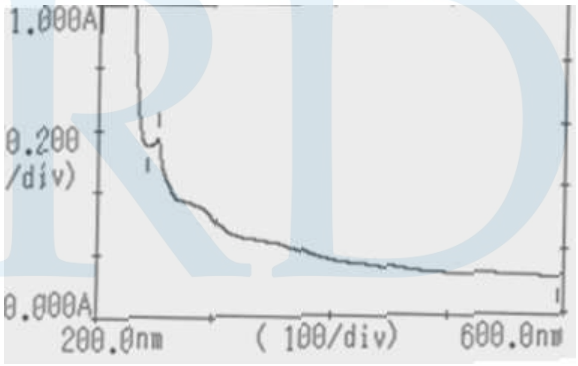


Fig.3: Sodium methoxide spectrum of compound I

When the shift reagent: sodium acetate was added to a methanolic solution of compound I a bathochromic shift diagnostic of a 7-OH function was observed (Fig.4).

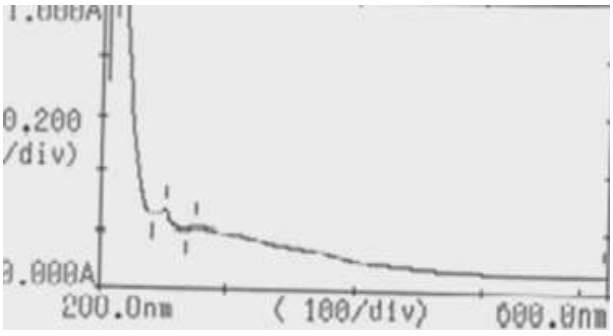


Fig. 4: Sodium acetate spectrum of compound I

The aluminium chloride spectrum (Fig.5) showed a bathochromic shift which is indicative of a B ring catechol system (the spectrum degenerated on addition of HCl- Fig.6). Since band I (Fig.2) is split into two peaks , this catechol was assigned positions 3` and 4` of the B ring[22].

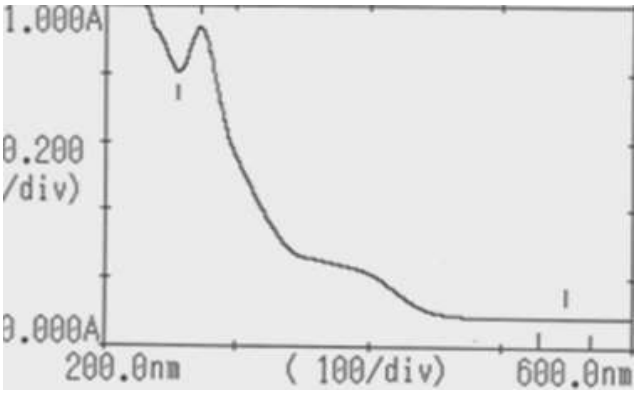


Fig. 5: Aluminium chloride spectrum of compound I

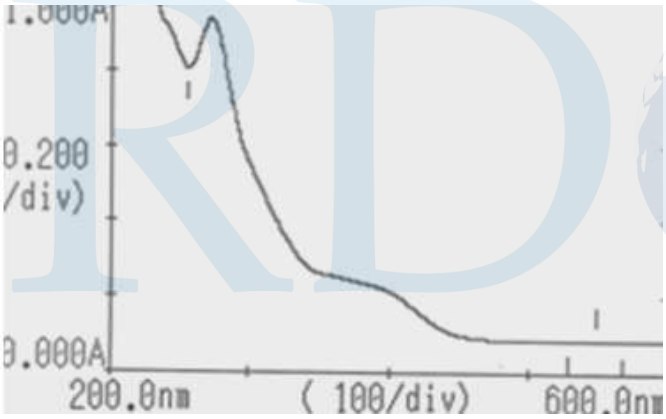


Fig. 6: Aluminium chloride/HCl spectrum of compound I

The ^{1}H NMR spectrum (Fig.7) exhibited a pattern characteristic of flavonoids.The signal at δ 1.23 is characteristic of a methyl group. The aliphatic proton at C_2 resonated downfield at 1.91ppm due to the electron-withdrawal effect of the oxygen bridge at position 1. The resonance at δ 3.93ppm is due to a methoxyl function which was assigned for C_3 on the basis of the retro Diels-Alder cleavage (Scheme I) and the absence of any multiplet characteristic of a C_3 methylene moiety. The signals at δ 6.20 and 6.68ppm account for C_6 – and C_8 –protons respectively. Usually the latter resonates downfield relative to the former proton due to the electron-withdrawal influence of the oxygen at position 1. The singlets at δ 7.37 and δ 7.95 ppm which are characteristic of two meta–coupled protons (J= 2.3) were assigned for $C_{2`}$ - and $C_{6`}$-

protons of the B ring.The C_5 proton ,and as anticipated , resonated well downfield due to the electron –withdrawal influence of the neighboring keto function at C_4.(signals at δ2.50 and δ3.30ppm are due to DMSO residual protons and DMSO water respectively).

The mass spectrum (Fig.8) gave m/z318 corresponding to(M^+ + 2H).Two important fragments corresponding to intact A and B rings, and resulting from a retro Diels-Alder fission (Scheme I), appeared at m/z 136 and m/z180 respectively. The appearance of such fragments lends evidence for the suggested substitution pattern for the A and B rings.

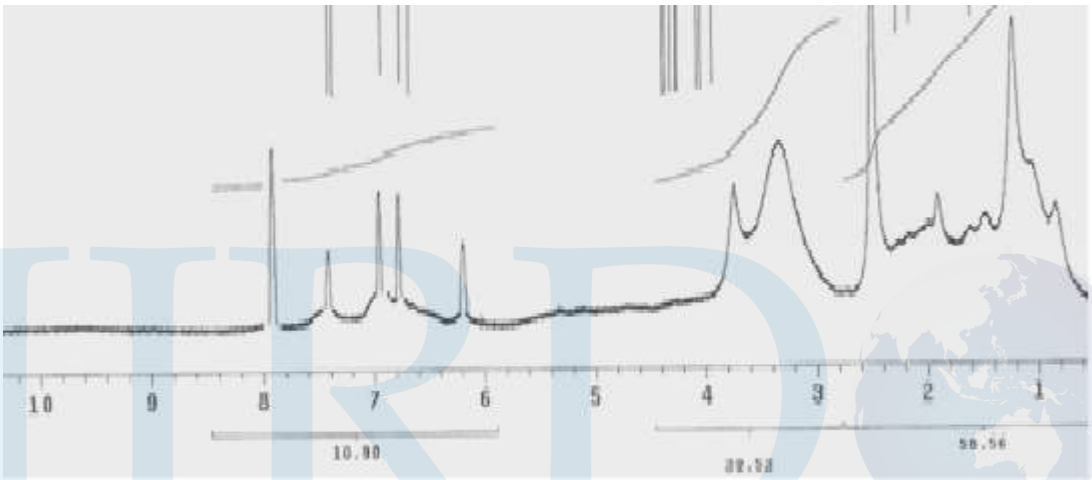


Fig.7:[1]HNMR spectrum of compound I

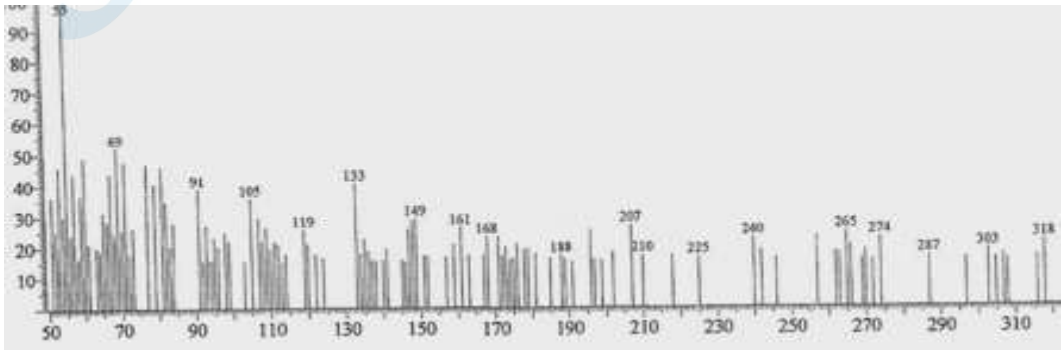
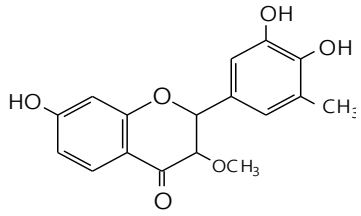


Fig.8: Mass spectrum of compound I

Comparison of the above spectral data with literature data gave the following structure for compound I:



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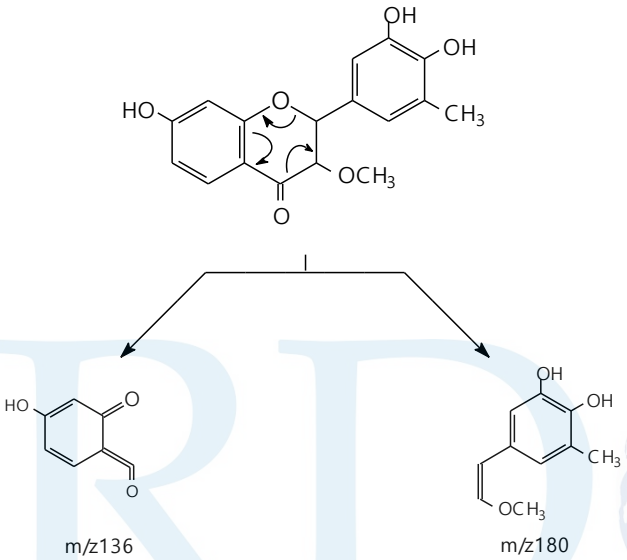

Scheme I: Retro Diels-Alder fission of compound I

3.2-Antimicrobial activity

Compound I and different fractions of *Geigeria alata* (ethanol, chloroform,n-butanol and ethyl acetate) were screened for their antimicrobial activity against six standard microorganisms(Table 1).The results are depicted in Table (2) .Results were interpreted in the following conventional terms : (<9mm: inative;9-12mm:partially active;13-18mm: active;>18mm:very active) .Tables (3) and (4) represent the antimicrobial activity of standard antibacterial and anifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 1: Test organisms

Ser. No	Micro organism	Type	Source
1	*Bacillus subtillus*	G+ve	ATCC 2836
2	*Staphylococcus aureus*	G+ve	ATCC 29213
3	*Pseudomonas aeroginosa*	G-ve	NCTC 27853
4	*Escherichia coli*	G-ve	ATCC 25922
5	*Aspergillus Niger*	fungi	ATCC 9736
6	*Candida albicans*	fungi	ATCC 7596

* NCTC. National collection of type culture, Colindale. England

*ATCC. American type culture collection, Maryland, USA

Table 2 : Antibacterial activity of comound I and different fractions

Fraction/ Comp.	Conc.(mg/ml)	Sa	Bs	Ec	Ps	Ca	An
Ethanol Ext.	100	--	--	--	--	--	14
Ethyl acetate Ext.	100	17	18	19	20	15	14
n-Butanol Ext.	100	14	13	13	--	--	10
Chloroform Ext.	100	11	11	13	11	11	13
Comp. I	100	13	14	--	--	-	--

Table 3 : Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-

	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 4 : Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	An	Ca
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

Sa.: *Staphylococcus aureus*
Ec.: *Escherichia coli*
Pa.: *Pseudomonas aeruginosa*
An.: *Aspergillus niger*
Ca.: *Candida albicans*
Bs.: *Bacillus subtilis*

The ethyl acetate fraction of *Geigeria alata* showed excellent antibacterial activity against all test microorganisms.However, the ethanol extract was inactive against the test bacteria ,but it gave good antifungal acivity against the fungus : *Aspergillus niger* . The chloroform and n-butanol fractions exhibited moderate activity against *Escherichia coli* (Table 2).

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