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ISOLATION AND CHARACTERIZATION OF A FALAVANONE FROM SUDANESE *DETARIUM SENEGALENSE* (J. F. GMEL.) STEM BARK AND BIOLOGICAL ACTIVITY OF DIFFERENT FRACTIONS

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ABSTRACT

Information on the bioactive constituents of medicinal plants used in Sudanese ethno-medicine is very scarce. Hence,this study was designed to investigate the flavonoids of *Detarium senegalense* which is widely used by local healers to treat an array of human disorders. Phytochemical screening of *Detarium senegalense* ethanolic extract revealed the presence of sterols, triterpenes, flavonoids, tannins, saponins, anthraquinones and cyanogenic glycosides. A Flavanone was isolated from stem bark and its structure was partially elucidated on the basis of its spectral data(IR,UV,^{1}HNMR and MS). Different fractions(chloroform, ethyl acetate, n-butanol and ethanol) were evaluated, *in vitro*, for antimicrobial activity against six standard human pathogens: two Gram positive (*Staphylococus aureus* and *Bacillus subtilis*), two Gram negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria and two fungal species (*Aspergillus niger*, *Candida albicans*) and significant results suggested a rationale for its traditional uses.

KEYWORDS: *Detarium senegalense*, Isolation, Flavanone, Antimicrobial Activity.

INTRODUCTION

In the family Fabaceae, *Detarium*, is a genus being represented by 8 species. In west African forests, only three species are found: *Detarium macrocarpum* **Harms**, *Detarium*

microcarpum **Guillemin** and **Perrottet** and *Detarium senegalense* **J.F. Gmelin**. These species are of pharmacological interest.[1]

Detarium senegalense **J. F. Gmel**. or Sweet detar is a species of plant in the Fabaceae family[1-4], subfamily: Caesalpinioideae and tribe: Detarieae. (Also placed in Leguminosae and Caesalpiniaceae).[1,2] It grows naturally in the drier regions of west and central Africa extending from Senegal and Gambia east to north-east tropical Africa and Sudan.[5,6] *Detarium senegalense* fruit seems to be a very healthful nutrient. It contains (Per 100 g): 27 mg calcium, 48 mg phosphate, 0.14 mg thiamin, 0.05 mg riboflavin and 1000–2000 mg ascorbic acid. Fruit pulp contains(per 100g): 2.8 mg iron, 0.6 mg niacin and about 1200 mg vitamin C.[7] Lesser quantities of other vitamins and minerals were reported.[8] Seeds are claimed to contain 12% protein and are rich in lysine-the rare amino acid- and tryptophan.[3] Stem bark and seeds were found to be rich in vitamins comprising(mg/100g): riboflavin 0.62 – 0.60, thiamin 0.14 – 0.27, niacin 2.06 – 8.11 and ascorbic acid 83-60.[9] The nutritional value of *Detarium senegalense* seeds seems to justify its use in the traditional treatment of skin diseases.[10]

Phytochemical studies of the seeds and stem bark revealed the presence of: alkaloids, flavonoids, tannins, phenols and saponins.[9] *Detarium senegalense* seeds contain a large amount of water-soluble, non-starch polysaccharide (s-NSP) suggesting important nutritional value. The main monosaccharide residues are:glucose, xylose, and galactose.[11]

The fruit pulp is edible, eaten raw or cooked. The seed is oily and edible and pounded seed is used as cattle feed. Seed flour is used traditionally in Nigeria as a emulsifying, flavouring and thickening agent in foods.[12] It is also employed as soup thickener.[13]

GC-MS analysis of the petroleum ether seed extract revealed the presence of ten constituents being dominated by oleic and linoleic acids (30.8 and 44.1% respectively). Hanan demonstrated[14] that the petroleum ether fraction exhibited significant antibacterial activity against a panel of human pathogens. Sowemimo *et al,*. claimed[10] antifungal activity against *Aspergillus flavus*, *Aspergillus niger* and *Penicillium notatum*.

A xyloglucan (a non - starch polysaccharide) extracted from *Detarium senegalense*, has considerable promise in the treatment of diabetes and hyperlipidaemia. The rheological and other properties of this component indicate a considerable commercial potential in the food,

drugs and chemical industries.[15,16] Recently There was considerable interest in the functionality of dietary polysaccharides starch and non-starch polysaccharides (NSP) with respect to prevention and treatment of disease such as diabetes, coronary heart disease, and arthritis.

Detarium senegalense is widely used in ethno medicine to treat an array of human disorders. Bark is claimed to treat dropsy, swelling and oedema. A decoctions of bark is used to treat digestive disorders, bronchitis, pneumonia and stomach –ache.[14,17,18], while bark powder is applied to wounds and burns. Bark pulp is eaten as a general tonic and in treatment of tuberculosis.[19.20] The bark is also used for headache, back-pain, sore throat and painful menstruation[17,21] and aqueous bark extract is used to cure indigestion.[17] Root decoctions are administered as anodyne and to treat intestinal disorders[20], convulsions[18] and anaemia.[22] Leaf decoction is said to treat fever, trypanosomiasis, dysentery, anaemia, conjunctivitis[18], arthritis, inflammations, fractures, boils and skin infections.[20] Fruit pulp is a treatment for kidney troubles, spinal tuberculosis, syphilis, cough, rheumatism and leprosy.[12,23] In one study a *Detarium senegalense* meal was shown to elicit significant reduction in plasma glucose levels in studied human subjects.[24] Seeds have been effective in controlling blood glucose levels in diabetic individuals.[10] Also they are taken as antidote against snake bites and arrow poison.[18] Seeds are also used by local healers as emetic. Root and fruit are used as painkillers.[12,23] Root and leaves decoctions are used in paralysis, meningitis and difficult delivery.[17] *In vivo* studies testified promising antidiabetic effect for gum[25]. Seeds, leaves and root decoctions are used as remedy for venereal diseases, intestinaal worms, diarrhea, urogenital infections, hemorrhoids, rheumatism, stomach ache, leprosy and malaria.[17,21]

Leaves have demonstrated antiviral activity against a number of human and animal viruses and the bark has shown antibacterial activity against many pathogenic bacteria, justifying the medicinal properties of the plant.[26] An anthocyanin alkaloid 2-methoxyamine 3,4,5,7-tetrahydroxyanthocynanidine has been isolated from the stem bark. *In vitro* antibacterial studies showed that the isolated compound successfully inhibited a panel of human pathogens.[19] Such results authenticate the use of *Detarium senegalense* in phytomedicine for the treatment of infections.

In continuation of our interest in the constituents of medicinal plants used in Sudanese traditional medicine, this study was designed to investigate the constituents of the Sudanese material of *Detarium senegalense* and to evaluate the antimicrobial activity of its fractions, hoping to establish a rationale for its ethno-medical uses.

MATERIALS AND METHODS

Plant Material

The stem barks of *Detarium senegalense* were collected from western Sudan. The plant was authenticated by direct comparison with a herbarium sample.

Solvents

Analytical grade solvents were used. Methanol -HPLC grade- was used for spectroscopic purposes (Loba, India).

Equipments

The ultraviolet lamp used in visualizing TLC plates and paper chromatography was a multiband UV λmax (254 / 365 nm) portable ultraviolet lamp, a product of Hanovia Lamps (6 watt S/Y and L/W).Ultraviolet absorption spectra were obtained in spectroscopic methanol on UV -Visible Spectrophotometer(Shimadzu).

Infrared spectra were generally obtained in potassium bromide (KBr) discs using Perkin-Elmer, FTIR, model 1600-Jasco. The electron impact ionization (EIMS) mass spectra were obtained on a solid probe using Shimadzu QP-class-500.

^{1}HNMR spectra were obtained on a Bruker AM 500 spectrophotometer (Germany) operating at 500 MHz in spectroscopic grade DMSO-d_6. The chemical shifts values are expressed in δ (ppm) units using (TMS) as an internal standard and the coupling constants (J) are expressed in Hertz (Hz).

METHODS

Stepwise procedure for use of shift reagents for UV

- The UV spectrum of the compound in methanol was first recorded.
- 3 drops of NaOMe reagent were added to the sample and the NaOMe spectrum was recorded, and after 8 minutes the NaOMe spectrum was re-recorded.

- 6 drops of $AlCl_3$ reagent were added to the fresh sample and the $AlCl_3$ spectrum was recorded, 3 drops of HCl were added and after mixing, the $AlCl_3$/ HCl spectrum was recorded.
- Powdered NaOAC was then added to the fresh sample, the mixture was shaked and the NaOAC spectrum was recorded. NaOAC/ H_3BO_3 spectrum was then recorded after adding H_3BO_3.

Preparation of plant extract for phytochemical screening

Powdered shade-dried stem bark(300g) of *Detarium senegalense* was macerated with 95% ethanol for 48hr. The solvent was removed *in vacuo* to give a crude extract used for phytochemical screening according to the method described by Harborne.[27]

Extraction of flavonoids

Powdered shade – dried stem bark of *Detarium senegalense* (2 kg) was macerated with 95% ethanol at ambient temperature for 48 hours. The solvent was removed under reduced pressure to give a crude extract.

Paper chromatography(PC)

The ethyl acetate fraction of *Detarium senegalense* stem bark was dissolved in methanol and applied to the Whatman paper (No. 3 mm – 46x 57cm), the bands were irrigated with BAW (n-butanol acetic acid-water, 5:2:6; v:v:v). The developed chromatograms were air-dried and examined under both visible and UV light (ʎ 366,245nm). The equivalent bands from each paper were then cut out, combined, cut into small strips and slurred with methanol. After several hours of contact with occasional shaking, the liquid was filtered and evaporated to dryness *in vaco* to afford compound I in chromatographically pure form.

Antimicrobial assay

In disc diffusion bioassay, different fractions (chloroform, ethyl acetate, n-butanol, ethanol) of *Detarium senegalense* stem bark were screened for their antimicrobial activity against four bacterial species, Gram-positive (*Staphylococus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and two fungal species **(***Aspergillus niger, Candida albicans*).

Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed on to nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

Preparation of fungal suspension

The fungal cultures were maintained on dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100ml of sterile normal saline, and the suspension was stored in the refrigerator until used.

Testing of antibacterial susceptibility

The paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 1999). Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extract. The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured.

Testing of antifungal susceptibility

The above mentioned method was adopted for antifungal activity, but instead of agar, dextrose agar was used. Samples were used here by the same concentrations used above.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening of *Detarium senegalense* ethanolic extract revealed the presence of sterols, triterpenes, flavonoids, tannins, saponins, anthraquinones and cyanogenic glycosides (Table 1).

Table 1: Preliminary phytochemical screening.

Phytochemical	***Detarium senegalense* extract**
Sterols	+ve
Triterpenes	+ve
Alkaloids	-ve
Flavonoids	+ve
Tannins	+ve
Saponins	+ve
Coumarins	-ve
Anthraquinones	+ve
Cyanogenic glycosides	-ve

The ethyl acetate fraction was purified by paper chromatography. After the usual workup, compound I was isolated from *Detarium senegalense* stem bark in chromatographically pure form.

Identification of compound I

The IR spectrum of compound I (Fig.1) showed ν(KBr)770(C-H,Ar.bending),1037(C-O), 1454, 1510(C=C, Ar.), 1647(C=O), 2920 cm^{-1} (C-H,aliph.).

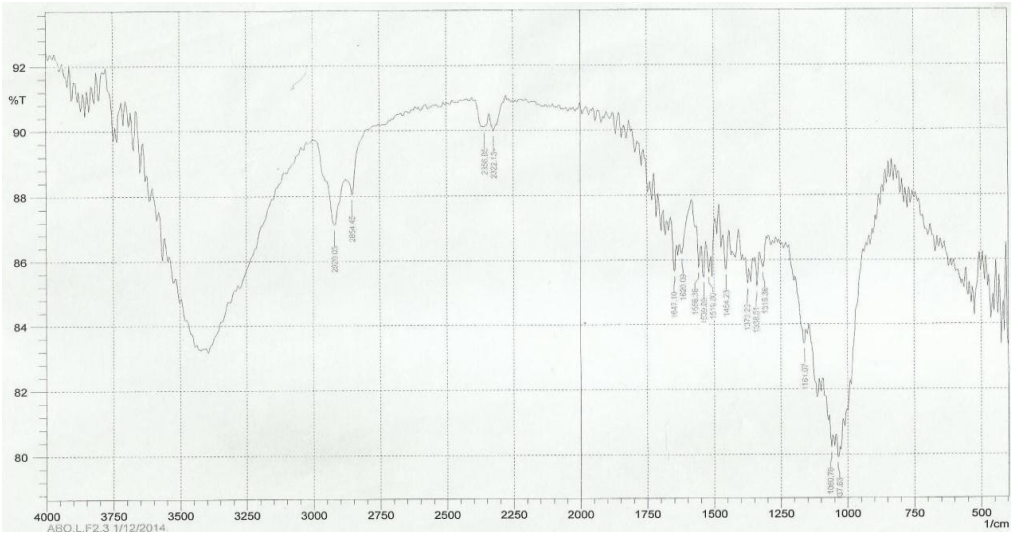


Fig.1: IR spectrum of compound I.

Compound I absorbs in the UV (Fig.2) at λ_{max}(MeOH)281nm.Such absorption is characteristic of : isoflavones , flavanones and dihydroflavonol.

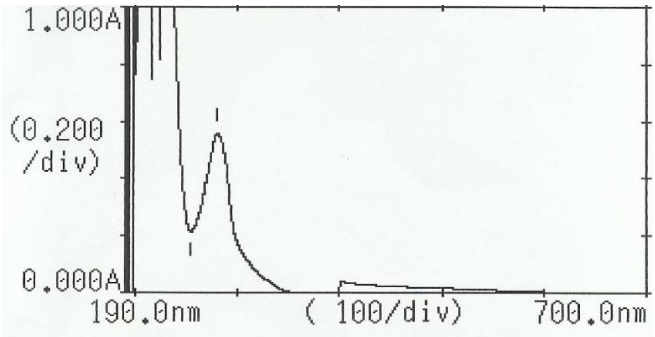


Fig. 2: UV spectrum of compound I.

No shoulder characteristic of isoflavones appeared in the UV spectrum(Fig.2) in the range 300-340nm. Furthermore , the sodium methoxide spectrum (Fig.3) did not reveal any bathochromic shift diagnostic of a 3-OH function which is a characteristic feature of dihydroflavonols.

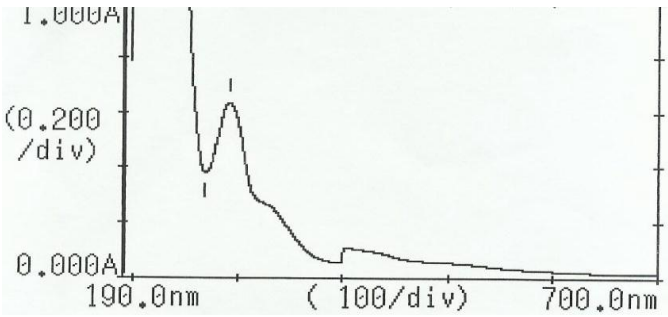


Fig.3: Sodium methoxide spectrum of compound I.

The ^{1}H NMR spectrum(Fig.7) showed a double multiplet at δ3.0-3.80 and δ5.20-5.80ppm which usually appear in the spectra of flavanones due to mutual spin-spin splitting of the magnetically unequivalent C_3 – protons . The double doublet which appears due to this splitting is further split by C_2 – proton into a pair of multiplets. Such data suggests that compound I is a flavanone.

When a methanolic solution of compound I was treated with sodium acetate no bathochromic shift was observed in band II (Fig.4). This indicates absence of a 7-OH function.

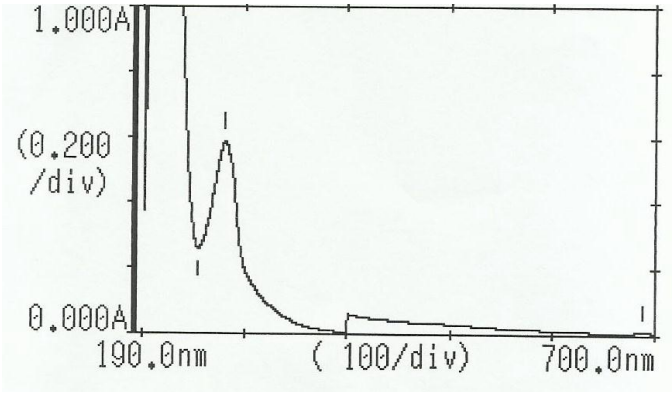


Fig .4: The sodium acetate spectrum of compound I.

The aluminium chloride spectrum of compound I (Fig.5)did not reveal any detectable bathochromic shift. This indicates absence of 5- and 3-OH functions as well as catechol moieties. Furthermore, the boric acid spectrum (Fig.6) testified the absence of catechol systems. It was devoid of bathochromic shifts.

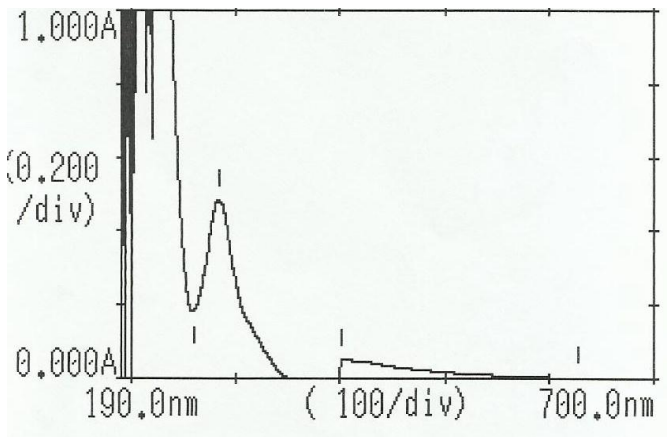


Fig.5: The aluminium chloride spectrum of compound I.

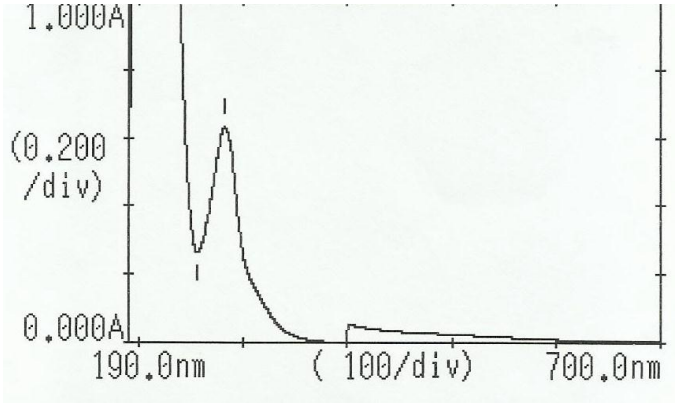


Fig.6: Boric acid spectrum of compound I.

The ^{1}HNMR spectrum (Fig.7) showed δ(ppm):1.20(6H) assigned for two methyl groups. The resonances at δ1.80 was attributed to an acetyl function, while the signal at δ4.20 accounts for a methoxyl group. The multiplet centered at δ3.60 accounts for a sugar moiety. Two multiplets characteristic of flavanones appeared at δ3.0-3.80 and δ5.20-5.80ppm.The multiplet at δ6.00-7.00δppm account for the aromatic protons.

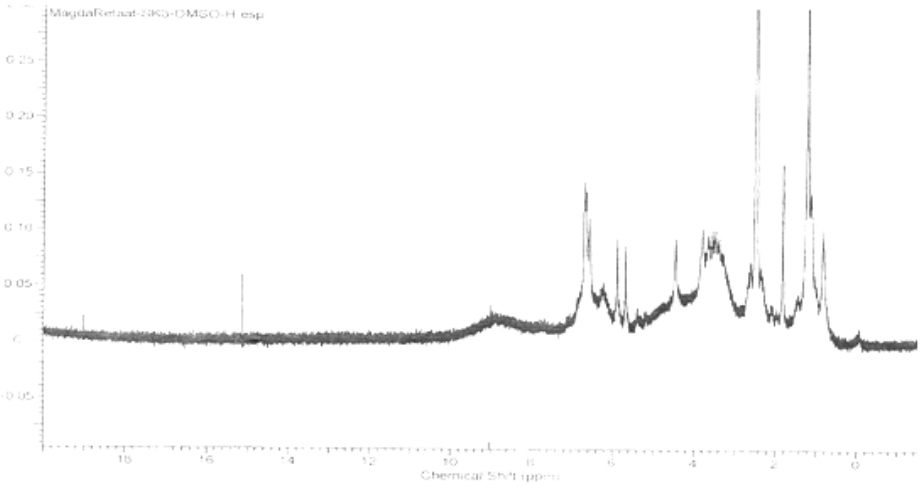
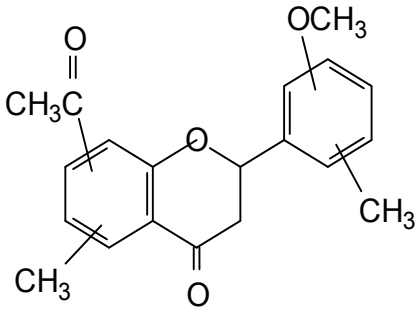
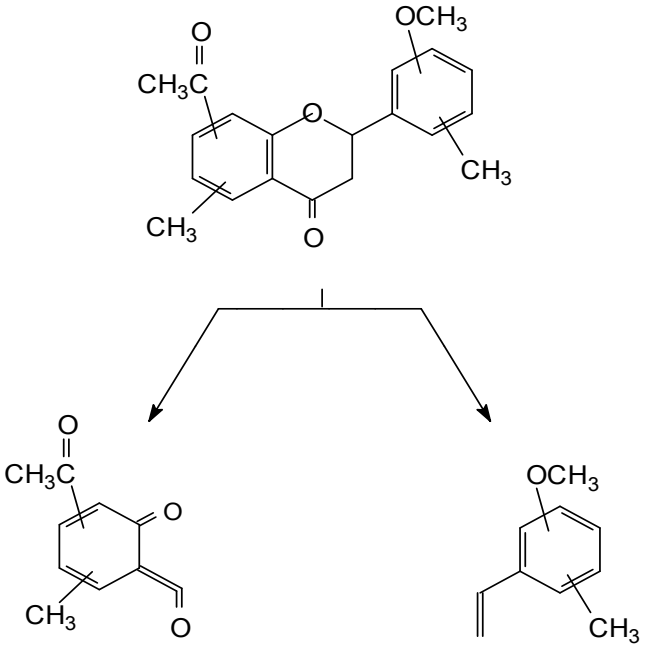


Fig.7: ^{1}HNMR spectrum of compound I

The mass spectrum gave m/z323 for M^{+}-H^{+}(aglycone). Two important fragments corresponding to intact A and B rings, appeared at m/z176 and m/z148 respectively. Such fragments lend evidence for the substitution pattern outlined in the following partial structure of compound I.



Compound I



m/z176 **m/z148**

Scheme II: Retro Diels-Alder fission of compound I

Antibacterial activity

In disc diffusion bioassay[28], chloroform, ethyl acetate, n-butanol and ethanolic fractions were screened for antimicrobial activity against six standard human pathogens. The average of the diameters of the growth of inhibition zones are depicted in Table (2). The results were interpreted in commonly used 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 antifungal chemotherapeutic agents against standard bacteria and fungi respectively**.**

Table 2: Antimicrobial Activity of *Detarium senegalense* fractions

Concentration		Antibacterial activity				Antifungal activity	
		Gram-positive		Gram -negative			
100mg/ml	Control Methanol	*Bs.*	*Sa.*	*Ec.*	*Pa.*	*Ca.*	*An.*
Ethanolic extract	0.0	23.5	21.5	21.5	25	15.5	19
Chloroform extract	0.0	22.5	16	15	15.5	20.5	20.5
Ethyl acetate fraction	0.0	24.5	26.5	23	22.5	18	22.5
n-Butanol fraction	0.0	20.5	21	23	23.5	19	21

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*

Different fractions of *Detarium senegalense* showed significant antimicrobial activity against test organisms as displayed in Table 2.

CONCLUSION

Detarium senegalense stem bark was screened for secondary metabolites. Paper chromatography allowed isolation of a flavanone from stem bark. On the basis of its spectral data, a partial structure was suggested for the isolate. Different fractions of *Detarium senegalense* stem bark were evaluated for antimicrobial activity against six standard human pathogens and significant results were obtained.

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