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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF A FLAVONE FROM SUDANESE *VITEX DONIANA* (SWEET)(VERBENACEAE) AND BIOLOGICAL ACTIVITY OF THE METHANOLIC FRACTION

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Abstract

From the leaves of *Vitex doniana* (Sweet) , a flavone (compound I) was isolated and characterized. The isolate was purified by different chromatographic techniques and identified via a combination of spectral tools(IR, UV, ^{1}HNMR and Mass spectroscopy). The methanolic fraction of *Vitex doniana* was evaluated(*in vitro*) for its antimicrobial potential against Gram negative (*Escherichia coli, Salomonella typhi* and *Pseudomonasa eruginosa*) and Gram positive (*Bacillus subtilis, Bacillus aureus and Staphylococcus aureus*) bacteria and the fungus *Candida albicans* .Promising results were obtained. *In vitro* antioxidant assay for the methanolic extract was conducted . Evaluation of the antioxidant activity was carried out by measuring the capacity of the extract against stable DPPH radical. The extract showed significant antioxidant activity.

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Introduction:-

Vitex doniana (**Sweet**)-black plum- (synonyms :*Vitex umbrosa*: **G. Don ex. Sabine**, *Vitex cienkowskii, Vitex chariensis*: **Chev**, *Vitex cuneata*:**Baker**, *Vitex paludoso*: **Vatke)** belongs to Verbenaceae family. Vitex comprise about 150 species distributed in tropics with a few species in temperate regions. Approximately 60 species occur in tropical Africa of which *vitex doniana* is most widespread(Adam,1966).

The variability of *vitex doniana* is remarkable, regarding its morphology as well as its habitat choice. Generally, *V. doniana* is a tree of evergreen. The most abundant and widespread genus occur in coastal savannah regions and lowland forests. The plant is distributed through Angola, Botswana, Ethiopia, Kenya, Namibia, Niger, Senegal, Somalia, South Africa, Sudan, Tanzania, Uganda and Zambia(Keay,1989).

The tree is planted for its sweet fruits. It contains vitamins A and B and can be made into a jam; fruit blackish pulp is rich in carbohydrates and minerals. In eastern Sudan, the fruits are roasted and used as substitute for tea. Leaves are often used as a herb for cooking. Young leaves and shoots are used as vegetable in some parts of Africa.

Vitex doniana parts have numerous applications in traditional medicine.The tree is used as treatment of several disorders and diseases which include rheumatism, hypertension, cancer, anemia, and the plant is said to improve fertility and is used to treat jaundice, leprosy, and dysentery(Orwa *et.al*.,2009) . Locally, the plant is used as insect repellent and insecticide.The root is used for gonorrhea and in treatment of stomach disorders, rickets, leprosy, diarrhea, dysentery, fevers, malnutrition and venereal diseases.

The leaves are used as an infusion for cold and flue. Hot aqueous extracts of the leaves are used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea and dysentery(Etta,1984;Irvine,1961) indicating that the leaves may possess anti-inflammatory and analgesic properties among others. Leaf sap is used as an eye drop to treat conjunctivitis and other eye complaints. Leaf decoction is applied externally against headache, stiffness, measles, skin rashes, fever, chicken pox and internally as tonic, febrifuge, and to treat respiratory tract diseases .The fruits are acidic, and is a source of essential minerals(Leung *et.al*.,1986) .The fruits are used as remedy for sores at the corners of the mouth and eyes, which are associated with vitamin A and B deficiencies . It is also used for diarrhea and dysentery. The twigs are used as chewing sticks for teeth cleaning.

Stem bark is used as remedy for leprosy, paralysis, epilepsy and convulsions. It is administered for ailments including diarrhea pulmonary troubles and skin rashes. Bark powder is added to water and taken to treat colic, and a bark extract is used to treat kidney diseases and to control bleeding after child-birth. The bark of *V doniana* is used in Kano traditional medicine as anti-epilepsy and against female sterility(Atawodi,2005).

Phytochemical screening of *Vitex doniana* showed variation in the distribution and type of secondary metabolites in the extracts. The ethanolic extract has shown the presence of alkaloid, flavonoids, reducing sugars and tannins..The antimalarial activity of an isolated compound(3-Ethyl-3,4,4a,5,6,6a,10a,11,12,12a-decahydro-1H-naphtho[2,3-g]quinolin-2-one) was found to be closer at all concentrations to the activity of the positive control (artesunate) (Mudi,2011) .

Hexane, ethyl acetate and methanolic extracts of stem bark were tested *in vitro* for antimicrobial potential using the well diffusion technique. The extract showed broad spectrum activity with zones of inhibition ranging from 19 to 24mm(Eghareba,2010).The petroleum ether extract of the leaves gave the highest activity with a zone of inhibition of 20mm against *Salmonella typhi.* Other extracts had zones of inhibition ranging from 4mm-14mm against almost all the tested organisms(Dauda *et.al*.,2011). Extract of the leaves demonstrated (Mudi,2011) a remarkable antimalarial activity at all concentrations. The most interesting anti-plasmodial activity was obtained with the methanolic extract.

Vitex doniana extracts showed significant antimicrobial activity against *S.typhi* (Dawang *et.al*.,2012) . Laboratory tests demonstrated the presence of high concentrations of progestogen-like compounds in *Vitex doniana* , thus natural consumption of *Vitex doniana* was a likely cause of the observed increases in progestogen(James *et.al*.,2007).

Ukwuani *et. al*.(2012) reported that the aqueous leaves extract of *Vitex doniana* has a significant antidiarrhoeal activity which supports its use in traditional herbal medicine practice and it was suggested(James *et.al.,*2010) that aqueous extract of *Vitex doniana* may exert anti-hepatotoxic effect against CCl_4-induced liver injury in animal model and that this effect is concentration- dependent.

Owolabi, *et. al*.(2011) suggested that the use of aqueous extract of *Vitex doniana* in the treatment of diabetes produce a significant ($p < 0.05$) antidiabetic and hepatoprotective effect..

Currently there is renewed interest in bioconstituents of plant species used in traditional medicine. So far, no report showed isolation of flavonoid compounds from Sudanese material of *Vitex doniana* . Hence, this work addressed interest in phytoconstituents of the Sudanese *Vitex doniana* which is used extensively by local healers in treatment of an array of human disorders.

Materials and Methods :-

Materials:-

Collection of plant material:-

Vitex doniana leaves were collected from Kordofan state,western Sudan and kindly authenticated by the Aromatic and Medicinal Plants Research Institute ,Khartoum , Sudan.

Test organisms:-
Methanolic extract of Vitex doniana leaves was screened for antibacterial and antifungal activities using the standard microorganisms shown in Table(1).

Table 1:- Test organisms.

Ser. No	Micro-organism	Type
1	*Bacillus subtilis*	G+ve
2	*Bacillus aureus*	G+ve
3	*Staphylococcus aureus*	G+ve
4	*Pseudomonas aeroginosa*	G-ve
5	*Escherichia coli*	G-ve
6	*Salomonella typhi*	G-ve
7	*Candida albicans*	fungus

Methods:-

Phytochemical screening:-
Preliminary phytochemical screening for secondary metabolites was conducted according to the method described by Harborne(1973).

Extraction of flavonoids:-
Powdered shade- dried leaves of *Vitex doniana* (2.5kg) were defatted with petroleum ether (40-60°C) and macerated at room temperature with 70% aqueous methanol . The methanol extract was evaporated under reduced pressure and temperature to afford 95g of a solid mass.

Isolation of flavonoids:-
A sample (150 g) dissolved in 50 ml aqueous ethanol (70%) was carefully applied to the top of a column (150x3.5 cm) containing 750g of polyamide 6S. Gradient elution started with water followed by H_2O/ EtOH mixtures of decreasing polarities at a flow rate of 1ml/ minute . The bands migrating along the column were traced under UV light during elution to note their characteristics and to control the fractionation process as well.Six fractionsl(each 100ml) were collected, dried *in vacuo* at ≈ 40°C and subjected to detailed investigations by two dimensional paper chromatography (2DPC).

Fraction IV was eluted with 60% EtOH and was found by (2DPC) to contain a mixture of flavonoids (dark purple spots on PC under UV light; turning yellowish with $AlCl_3$). Among the mixture one constituents (**I**) existed in appreciable concentration.

A sephadex column fractionation of fraction IV, using H_2O/EtOH mixtures for elution led to desorption of sub-fraction (i) which was collected and dried *in vacuo*. 2DPC revealed the presence of component (I) . Repeated preparative PC of this sub-fraction on Whatman Paper(No. 3mm) and irrigation with BAW afforded chromatographically pure sample of compound (**I**) .

Antimicrobial assay:-

Preparation of bacterial suspensions:-
One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°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 a suspension containing about 10^8-10^9colony forming units per ml. The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism 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 in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dry nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

Preparation of fungal suspensions:-
Fungal cultures were maintained on dextrose agar incubated at 25°C for four days. The fungal growth was harvested and 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 , with some minor modifications, to assess the antibacterial activity. (2ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°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 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 one of the halves was designed for one of the test solutions. Separate Petri dishes were designed for standard antibacterial chemotherapeutics(ampicillin and gentamycin).

The agar discs were removed, alternate cups were filled with 0.1 ml samples of test solution using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentrations of the standard chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in triplicates and averaged.

Results and Discussion:-

Phytochemical screening:-
Results of phytochemical screening of *Vitex doniana* leaves are depicted in Table 2.

Table 2:- Phytochemical screening of the leaves of *Vitex doniana*.

Constituents	*V. doniana*
Crystalline sublimate	-
Volatile matter	-
Carbohydrates and/or glycosides	+
Steroids and/or triterpenes	+
Flavonoids	++
a-aglycones b-glycosides	++
Tannins	-
Anthraquinones	-
a-free b-combined	-
Alkaloids and/or nitrogenous compounds	+
Saponins	++
Coumarins	+

++ = Present in a high concentration ; + = Present in a moderate concentration ; - = Absent

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Identification of compound I:-
The UV spectrum of compound I (Fig.1) showed λ_{max}(MeOH) 255,348nm . Such absorption is characteristic of flavones(Harborne,1998; Mabry,*et.al*.,1972).

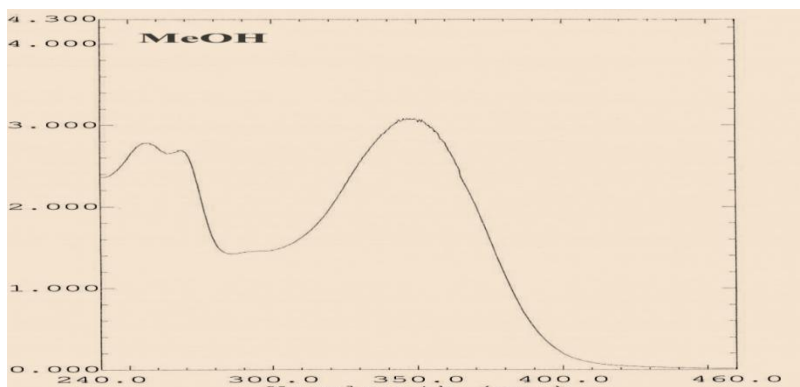


Fig. 1:- UV spectrum of compound I

The hydroxylation pattern of this flavone was investigated via UV shift reagents(Mabry *et.al.*,1972): sodium methoxide, sodium acetate, aluminium chloride and boric acid.

The sodium methoxide spectrum(Fig.2) revealed a 50nm bathochromic shift in band I without decrease in intensity indicating a 4`-OH function(Harborne,1998).

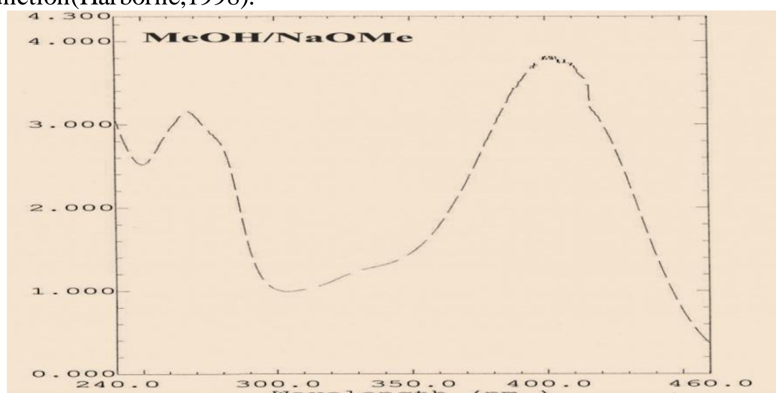


Fig 2:- Sodium methoxide spectrum of compound I

The shift reagent $AICI_3$ gave a bathochromic shift (69nm) indicating a catechol moiety(Figures 3 and 4).Such hydroxylation pattern is further confirmed by the boric acid spectrum .Boric acid spectrum of compound I (Fig.5) revealed a 25nm bathochromic shift in band I indicating a B ring catechol moiety. Since band II in the methanolic spectrum (Fig.1) consists of two peaks the catechol is located at $C_{3`}$,$C_{4`}$(Harborne,1998; Mabry *et.al.*1972) .

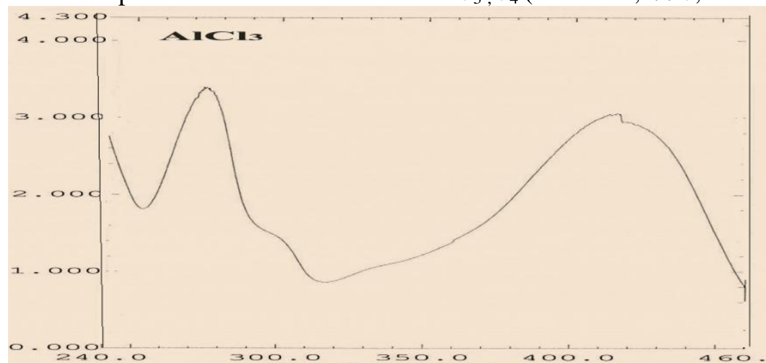


Fig.3:- Aluminium chloride spectrum of compound I

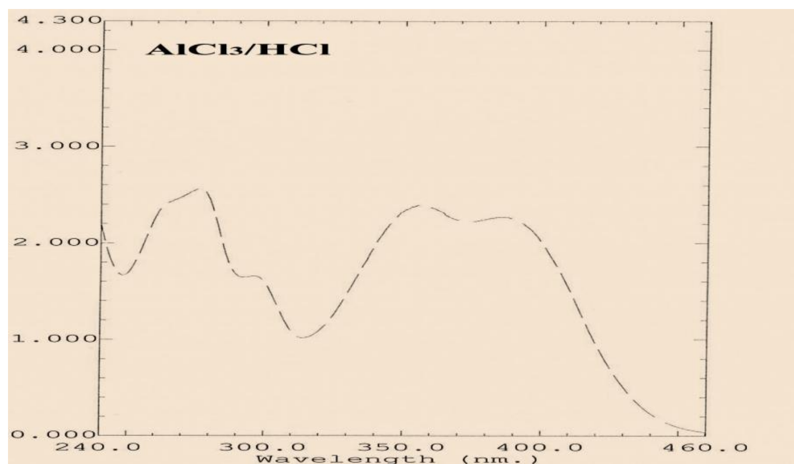


Fig.4:- Aluminium chloride/HCl spectrum of compound I.

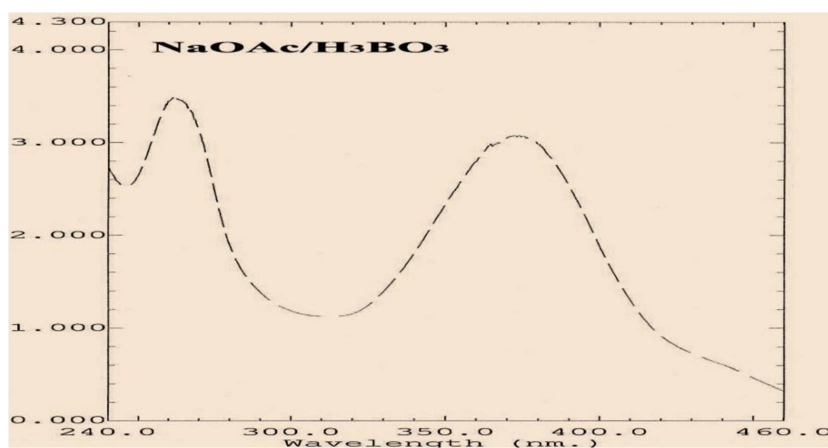


Fig.5:- Boric acid spectrum of compound I

A fifteen nm bathochromic shift in band II was observed(Fig.6) when sodium acetate was added to a methanolic solution of compound I indicating a free 7-OH(Mabry *et.al.*,1972).

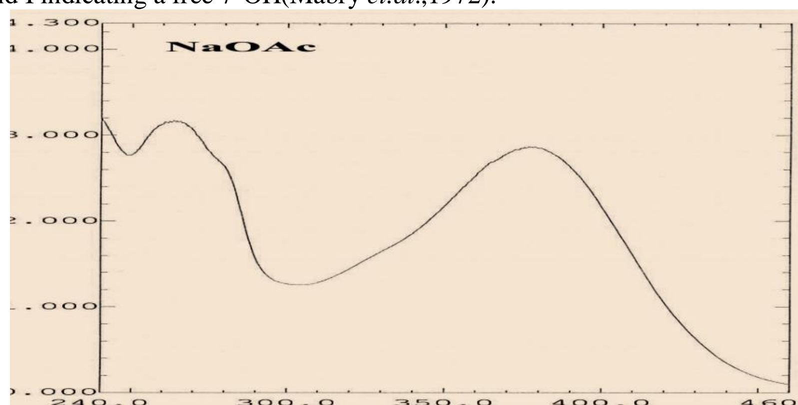


Fig. 6:- Sodium acetate spectrum of compound I

The UV spectral data of compound I are summarized in Table (3).

Table 3:- UV spectral data of compound I

	λ_{max} (nm)
MeOH	255, 348
NaOMe	267, 281, 332sh, 407
$AICI_3$	273, 301,333,417
$ALCI_3$ / HCI	276, 299sh, 353, 391
NaOAc	265, 378
NaOAc / H_3BO_3	259, 264, 373

The ^{1}HNMR spectrum(Fig.7) gave a resonance at δ3.40(5H) which accounts for sugar protons . The anomeric proton resonated downfield relative to the rest of the sugar protons at δ4.65ppm.No signal for the vinylic proton of C_3 was observed in the spectrum and this postion seems to be the site of glycosylation. Acid hydrolysis followed by UV studies using the shift reagent sodium methoxide confirmed a C_3-glycosylation. Acid hydrolysis followed by paper chromatography using authentic samples indicated the presence of glucose.The signal at δ1.10(3H) accounts for a methyl group,while the resonance at δ3.46(6H) was attributed to two methoxyl groups.On the basis of the retro Diels-Alder fission (Scheme I), one of these methoxyls was assigned to A ring , the other one exists in B-ring on the same basis(see scheme I). The double doublets at δ6.21 and δ 6.81ppm are characteristic of C_6 - and C_8 -protons respectively. The resonance at δ7.49ppm was assigned for $C_{2`}$ and $C_{6`}$- protons which usually appear as a singlet in case of a $C_{3`,4`,5`}$- oxygenation(Harborne,1989) .The mass spectrum (Fig.8) gave m/z360 for the molecular ion of the aglycone .The glycoside rarely give discernible molecular ions(Mabry *et.al.* ,1973;Harborne,1989) .Other important fragment (m/z137,149 and m/z181)resulting from the retro Diels-Alder fission (scheme I) of the aglycone were also recorded in the electron beam.These fragments which result from intact A and B rings site evidence for the suggested substitution pattern in the flavone nucleus.

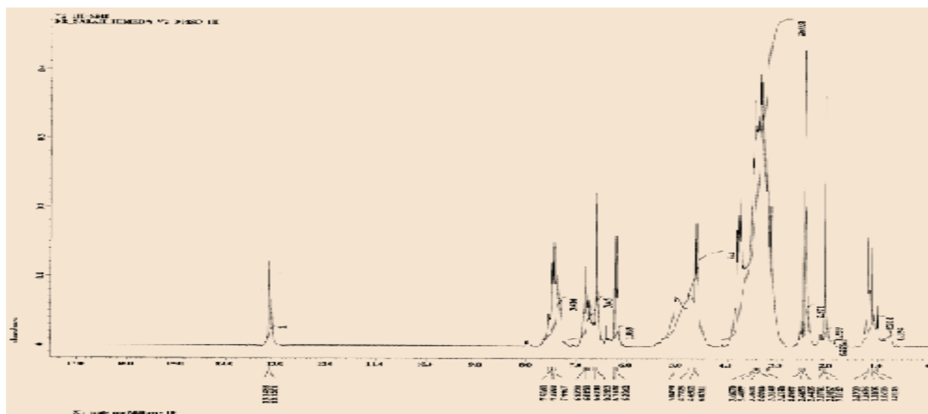


Fig. 7:- ^{1}H NMR spectrum of compound I

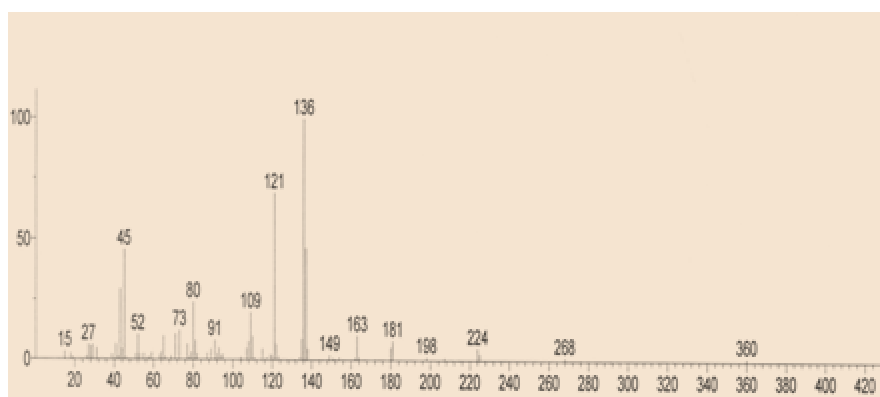
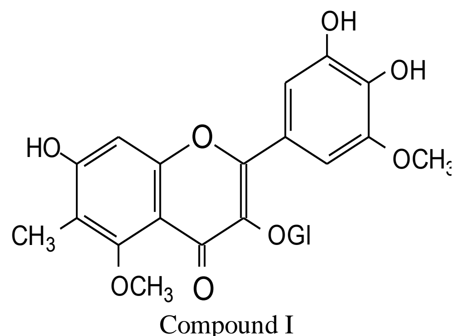


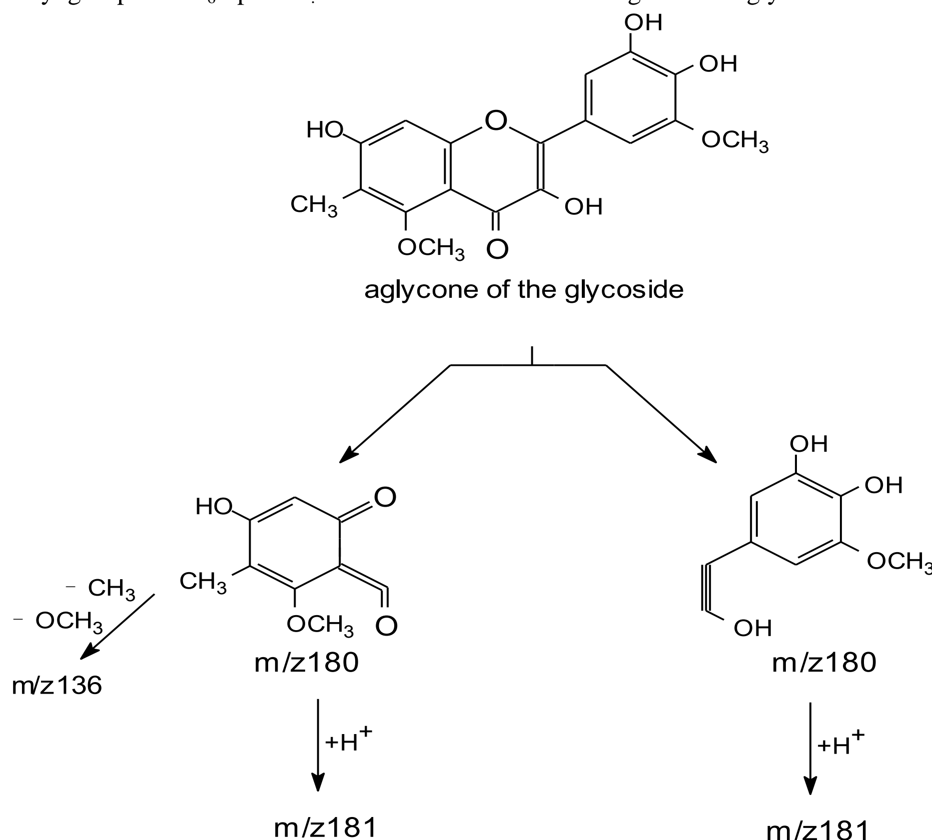
Fig. 8:- Mass spectrum of compound I

Comparison of the above cumulative data with literature data resulted in the following structure for compound I:



Compound I

The C_6 methylation was dictated by HSQC data which indicated direct connectivity of the methyl function to C_6 .The assigment of the methoxyl functions at C_5 and $C_{5'}$ was based on ^1H-^1H COSY NMR experiments which indicated long range coupling between a methoxyl function and C_6 – methyl group ,and another long range coupling between a methoxyl group and $C_{6'}$ -proton. The retro Diels-Alder cleavage of the aglycone is shown in scheme I.



Scheme I:- Retro Diels – Alder fission of aglycone of compound I

Biological activity:-

Antimicrobial activity:-

The antimicrobial response of the methanolic extract of *Vitex doniana* is depicted in Table 4.The results were interpreted in terms of the commonly used terms ; <9mm: inative;9-12mm:partially active;13-18mm: active;>18mm:very active) .

Diameters of the growth inhibition zones for standard chemotherapeutic agents are depicted in Tables (5) and (6). The methanolic extract exhibited significant antibacterial activity against Gram negative bacteria *Pseudomonas aeruginosa*.It also showed activity against Gram negative bacteria *Salmonella typhi* and the fungus *Candida albicans*. Thus this extract is a promising candidate for future optimization.

Table 4:- Antimicrobial activity *Vitex doniana* methanolic extract

Microorganism	Gram	Methanolic extract(100mg/ml)
Bacilus aureus	+ve	7
Bacilus subtilis	+ve	-
Staphylococcus aureus	+ve	-
Escherichia coli	-ve	-
Pseudomonas aeruginosa	-ve	20
Salomonella typhi	-ve	10
Candida albicans	Fungus	15

Table 5:- Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

Drug	Conc. nmg/ml	Bs.	Sa.	Ec.	Sa.
Ampicillin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	-
	20	22	18	18	-
	10	17	14	15	

Table 6:- Antifungal activity of a 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*
- Sa.*: Salomonella typhi*
- An.*: Aspergillus niger*
- Ca.*: Candida albicans*
- Bs.: *Bacillus subtilis*

Antioxidants activity of *Vitex doniana* methanolic extract:-

Evaluation of the antioxidant activity was carried out by measuring the capacity of the methanolic extract against stable DPPH radical**.**The methanolic extract of *Vitex doniana* showed high value for absorbance inhibition at a concentration of 64.2 µg/ml (Table 7 and Fig.9). It could be concluded from Figure 9 that the *Vitex doniana* extract showed high antioxidant capacity which is comparable to the value exhibited by the positive reference.

Table 7:- Antioxidant activity of the methanolic extract of *Vitex doniana.*

µl of the methanolic extract added to 3ml DPPH	Conc.(µg/ml)	Ascorbic acid	*Vitex doniana* extract
0	0	0	0
1	0.8	38.5	3.6
3	2.5	66.3	5.7
7	5.8	88.6	14.3
10	8.3	94.4	23.3
19	15.8	98.1	27.1
38	31.7	98.2	62.5
77	64.2	98.3	91

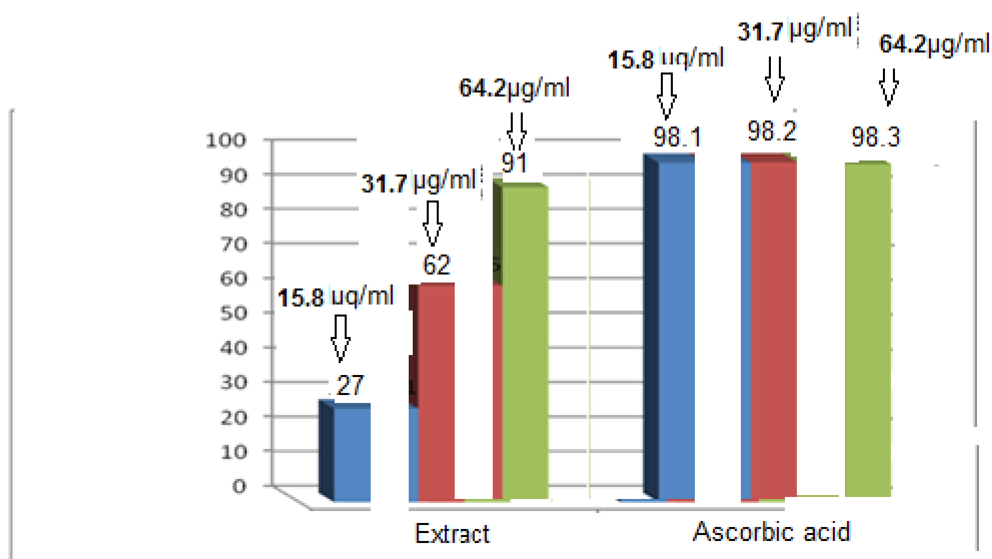


Fig.9: Antioxidant activity of Vitex doniana methanolic extract compared to ascorbic acid

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