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ISOLATION AND CHARACTERIZATION OF A DIHYDROCHALCONE FROM SUDANESE *BORASSUS AETHIOPIUM* (MART) STEM BARK AND BIOLOGICAL ACTIVITY OF ETHANOLIC EXTRACT

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ABSTRACT

Information on the constituents of medicinal plants used in Sudanese traditional medicine is very scarce. Hence,this study was set to investigate the phenolics of the medicinally important species *Borassus aethiopium* which is widely used in ethnomedicine to treat an array of human diseases. Phytochemical screening of *Borassus aethiopium* bark ethanolic extract revealed the presence of sterols, triterpenes, flavonoids, tannins, saponins and glycosides. A dihydrochalcone was isolated from stem bark and its structure was partially elucidated on the basis of its spectral data(IR,UV,^{1}HNMR and MS). The isolate was evaluated, *in vitro*, for its 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 were obtained.

KEYWORDS: *Borassus aethiopium*,Phytochemical Screening, Isolation, Dihydrochalcone, Antimicrobial Activity.

INTRODUCTION

Flavonoid compounds are one of the most analyzed group of secondary metabolites in higher plants. The main reason for the interest in flavonoids is that they are major constituents of plant pigments. Anthocyanins, a flavonoid subclass, have been of special interest because of

their ability to confer red, orange, blue, and purple coloration to leaves, flowers, and fruits.[1] As pigments, flavonoids have facilitated the testing of hypotheses related to Mendel's law and transposable elements. Flavonoids have been the focus of attempts to modify flower color by genetic engineering.[2] Some flavonoids have been reported to possess a variety of biological activities, including antiallergic, antiinflammatory, antiviral, antiproliferative, and anticarcinogenic activities, in addition to having effects on mammalian metabolism.[3,4]

Flavonoids have received considerable attention because of their beneficial effects as antioxidants in the prevention of human diseases such as cancer and cardiovascular diseases, and some pathological disorders of gastric and duodenal ulcers, allergies, vascular fragility, and viral and bacterial infections.[3] They contribute to the antioxidant properties of green vegetables, fruits, olive and soybean oils, chocolate, and teas.[5] Several mechanisms by which flavonoids play an important role in cytotoxicity have been identified. Antitumour activity of several flavonoids (pinostrobin, quercetin, myricetin, morin) is attributed to their efficiencies to inhibit topoisomerase I and II.[6.7] Flavonoids might slow down cell proliferation as a consequence of their binding to estrogen receptor.[8-12]

There is also interest in using them as drugs or dietary supplements because of their strong antioxidant activities. In plants, flavonoids have several functions including attracting insects for pollination and dispersal of seeds, acting in defense systems (e.g., as UV-B protectants and phytoalexins), signaling between plants and microbes, and regulating auxin transport. Many of these functions cannot occur unless flavonoids are properly localized within the cells.[13,14]

Borassus aethiopum(Mart) is a tropical plant in family Arecaceae widely distributed across the African continent . The trunk is used in construction purposes.[15] The fruit contains provitamin A , vitamin C and sugars.[16] Different parts of the plant are used traditionally to treat an array of human disorders including sexually transmitted diseases, fungal and viral infections particularly measles.[17] The root is used in treatment of asthma [15] while the flowers are used to treat impetigo. The antipyretic potential of this species has also been demonstrated.[18] It was claimed that the extract of the germinating fruit shoot has an anabolic effect of androgens.[19] Some studies indicated effective anthelmintic activity.[20,21] Seed coat methanolic extract has shown free radical scavenging action.[22] *In vivo* antimicrobial activity of a dichloromethane-methanol(1:1;v:v) extract of the male inflorescences of *B. aethiopum* was reported.[23]

MATERIALS AND METHODS

MATERIALS

plant material

Stem barks of *Borassus aethiopium* were collected in August 2015 from Nyala -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 paper chromatography was a multiband UV λ_{max} (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 a Shimadzu UV -Visible Spectrophotometer.Infrared spectrum was obtained in potassium bromide discs using a Perkin-Elmer, FTIR, model 1600-Jasco. The electron impact ionization (EIMS) mass spectrum was obtained on a solid probe using Shimadzu QP-class-500.^{1}HNMR spectrum was 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

Preperations of reagents for phytochemical screening

Different reagents used for phytochemical screening were prepared according to the method described by Harborne.[24]

Preparation of plant extract for phytochemical screening

(100 g) of powdered shade- dried plant material were macerated with 95% aqueous ethanol until exhaustion. This prepared extract(PE) was used for phytochemical screening.

Phytochemical screening

The prepared extract of *Borassus aethiopium* stem bark was screened for major secondary constituents according to the method described by Harborne.[24]

Test for unsaturated sterols and for triterpenes

(10 ml)of the (PE) of was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the colouring materials. The residue was then extracted with 10 ml chloroform. The chlorform solution was dehydrated over anhydrous sodium sulphite.(5 ml) portion of the solution was mixed with(0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml of filtrate a fragment of magnesium ribbon was added, shaked and then few drops of concentrated hydrochloric acid were added.
- To 3 ml of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml of the filtrate few drops of potassium hydroxide solution were added.

Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated. Few drops of Mayer reagent were added.

Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtered. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled , filtered and the volume adjusted to 10 ml with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

Test for Saponins

(1 g) of dried powdered plant material was placed in a test tube. (10 ml) of distilled water were added and the tube was stoppered and vigorously shaked for about 30 seconds, and allowed to stand . A froth that persisted for one hour is a positive test.

Stepwise procedure for use of shift reagents for UV

- The UV spectrum of the isolated flavonoid , in methanol, was first recorded.

- 3 drops of NaOMe reagent were added to the sample and the NaOMe spectrum was recorded.
- 6 drops of $AlCl_3$ reagent were added to a methanolic solution(1ml) of compound I 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 added to a methanolic solution(1ml) of compound I, the mixture was shaked and the NaOAC spectrum was recorded. NaOAC/ H_3BO_3 spectrum was then recorded after adding H_3BO_3.

Isolation of flavonoids

Powdered shade- dried stem barks of *Borassus aethiopium* were macerated with 95% ethanol at room temperature for 48 hours. The crude extract(4g) was mounted on top of a silica gel(400g) column and then eluted with methanol: ethyl acetate in order of increasing polarity starting with 4:1; 1:1(ethyl acetate:methanol) and ending with methanol. The methanol fraction was rich in phenolics. It was applied on Whatman paper (No. 3 mm – 46x 57cm) as narrow strips. The bands were irrigated with BAW (n-butanol- acetic acid-water; 4:2:5;v:v:v; upper layer). 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 and cut into small strips and slurred with methanol. After several hours of contact, with occasional shaking, the liquid was evaporated *in vacuo* to dryness. In this way compounds I was isolated from stem bark of *Borassus aethiopium* in chromatographically pure form.

Antimicrobial assay

Borassus aethiopium stem bark was screened for 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*). The cup plate agar diffusion bioassay was used.

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 suspensions were stored in the refrigerator until used.

Testing of antibacterial susceptibility

The cup plate agar diffusion method was used to screen the antimicrobial activity of plant extract 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 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

The ethanolic extract of *Borassus aethiopium* stem bark was screened for major secondary metabolites and the results are depicted in Table 1.

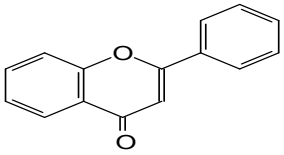
Table 1: Phytochemical screening of *Borassus aethiopium* stem bark.

Constituents	*Borassus aethiopium* extract
Alkaloids	**+ve**
Steroids	**+ve**
Glycosides	**+ve**
Triterpenes	**+ve**
Flavonoids	**+ve**
Tannins	**+ve**
Saponins	**+ve**

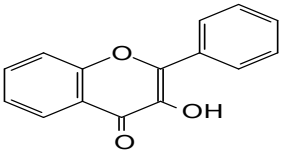
Identification of compound I

Compound I was isolated as yellow powder from the stem bark of *Borassus aethiopium*. The structure was partially elucidated via a combination of spectral techniques(UV,IR,^{1}HNMR an MS).

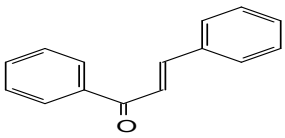
The UV spectrum of flavonoids can differentiate between those flavonoids with unsaturation at 2 and 3 positions(flavanones,flavonols,chalcones and aurones) and those classes lacking such unsaturation like flavones,dihydrochalcones,dihydroflavonols and isoflavones.



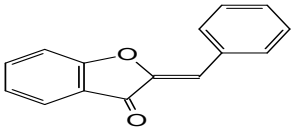
Flavone



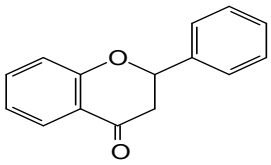
Flavonol



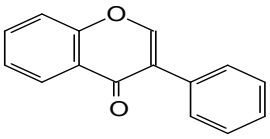
Chalcone



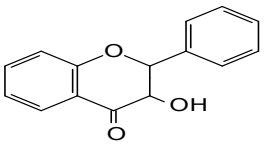
Aurone



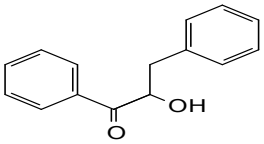
Flavanone



Isoflavone

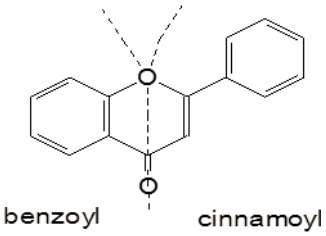


Dihydroflavonol



Dihydrochalcone

Flavonoids with conjugation between A and B rings show two absoption bands in the UV spectrum: band I (due to cinnamoyl chromophore) and band II (due to benzoyl chromophore).



The UV spectrum of compound I gave λ_{max} (MeOH) 206nm (Fig. 1) a pattern which is characteristic of dihydrochalcones.

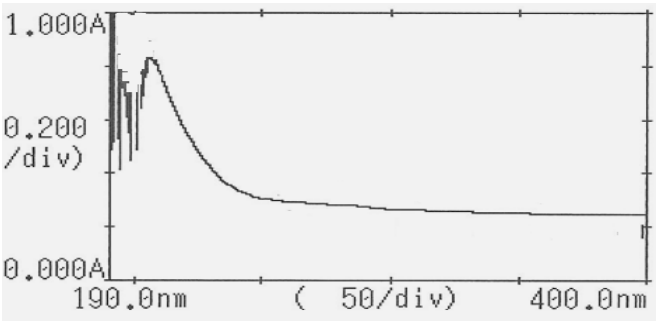


Fig.1: UV spectrum of compound I

The UV shift reagents (sodium methoxide, sodium acetate, aluminium chloride and boric acid/sodium acetate) can provide characteristic bathochromic shifts diagnostic of specific hydroxylation pattern. Sodium methoxide is strong base. It is diagnostic of α-OH and 4 –OH groups of dihydrochalcones. In both cases it induces a bathochromic shift, but with decreased

intensity in case of α-OH function.[25] Addition of sodium methoxide to a methanolic solution of compound I, induced a 10nm bathochromic shift without decrease in intensity indicating a 4-OH function(Fig.2)

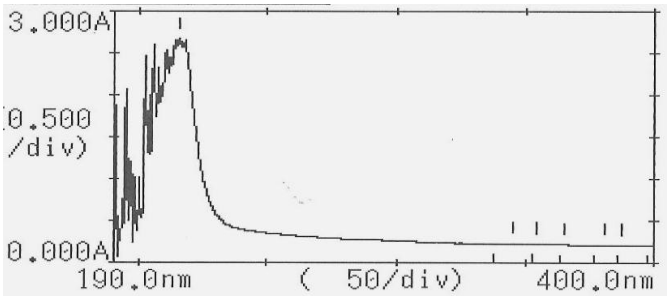


Fig 2: Sodium methoxide spectrum of compound I.

The sodium acetate(Fig.3) gave a 12 nm bathochromic shift with increase in intensity diagnostic of a 4 and a 4`-OH functions of dihydrochalcones.[25]

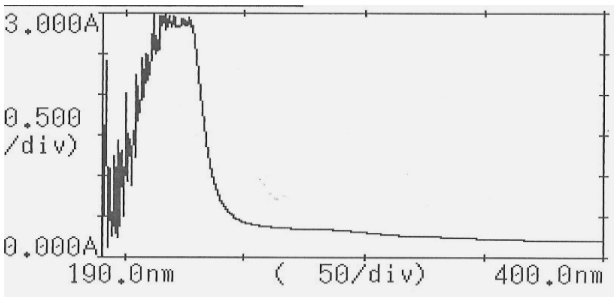


Fig 3: The sodium acetate spectrum of compound I

The aluminium chloride spectrum(Fig.4) did not reveal any detectable bathochromic shift indicating absence of α-OH, 2`-OH functions as well as catechol systems.[25]

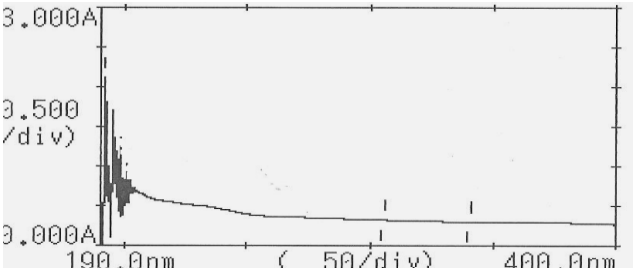


Fig 4: Aluminium chloride spectrum of compound I.

Aluminum chloride is a useful diagnostic tool for α- and 2`-OH groups of dihydrochalcones as well as catechol moieties .Catechols form acid – labile complexes with aluminium chloride , while the α-OH and 2`-OH afford acid – stable complexes.[25]

The ^{1}H NMR spectrum (Fig.5) showed amultiplet at δ 0.95-1.60(10H) assigned for two methyl functions and two methylene moieties. The resonance at δ3.80(6H) accounts for two methoxyl functions. The multiplet at δ6.40-7.00 ppm was assigned for the aromatic protons.

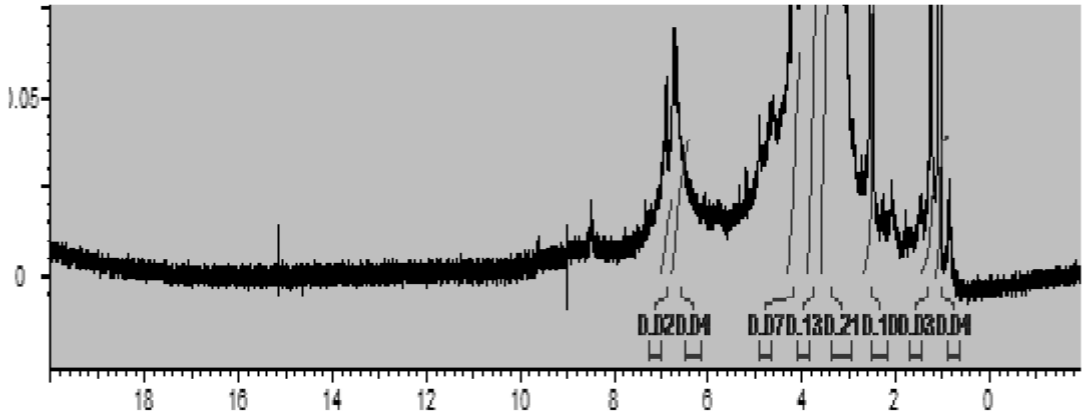
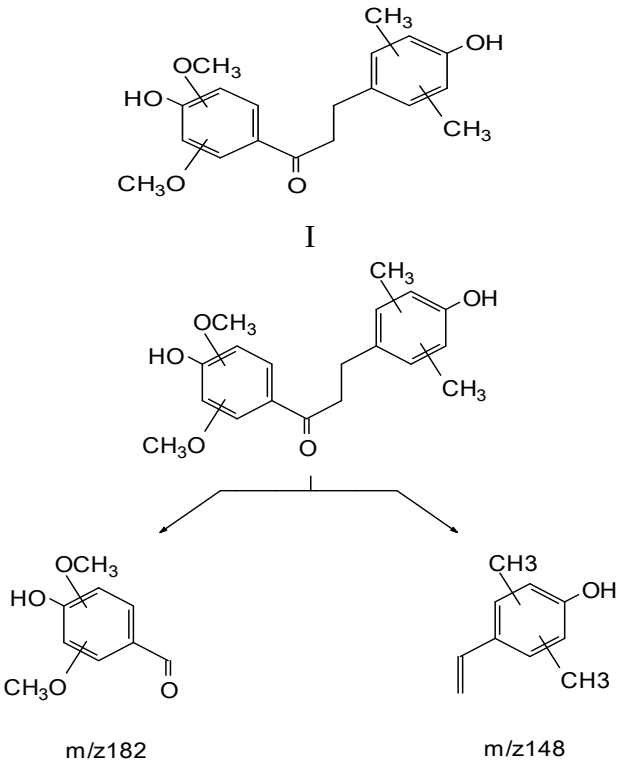


Fig.8: ^{1}H NMR spectrum of compound I

The mass spectrum gave m/z 332 for(M^{+}+ $2H^{+}$). Other important fragments resulting from intact A and B rings appeared at m/z182 and m/z148 respectively (Scheme I). This pattern of fission suggests two methoxyl functions in ring A and two methyls in ring B.

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



Scheme I: Fragmentation pattern of compound I

Antimicrobial activity

In cup plate agar diffusion assay , the ethanolic extract of *Borassus aethiopium* stem bark was 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 *Borassus aethiopium*. stem bark.

Concentration		Antibacterial activity				Antifungal activity	
		Gram-positive		Gram - negative			
mg/ml	Control Methanol	*Bs.*	*Sa.*	*Ec.*	*Pa.*	*Ca.*	*An.*
100	0.0	20	18	19	18	15	18
50	0.0	20	17	17	20	14	13
25	0.0	19	15	18	20	13	12

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 ethanolic extract of *Borassus aethiopium* stem bark showed significant antibacterial activity against test organisms specially at doses of 100 and 50mg. The extract also gave significant inhibition against the fungal species *Aspergillus niger* at 100mg/ml(Table 2).

CONCLUSION

Borassus aethiopium stem bark was screened for major secondary metabolites. Paper chromatography allowed isolation of a dihydrochalcone from stem bark. On the basis of its spectral data , a partial structure was suggested for the isolate. The ethanolic extract of *Borassus aethiopium* was evaluated for antimicrobial activity against six standard human pathogens and significant results were obtained.

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