

Isolation and Partial Characterization of a Dihydrochalcone From *Amomum subulatum* and Antimicrobial Activity of the Aqueous Extract

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Abstract

Amomum subulatum has been used since ancient times as spice. It is a herb, widely cultivated for its economic value. *Amomum subulatum* is astringent, stomachic, alexipharmic and stimulant. The oil is used traditionally against a wide array of human disorders.

In this study the fruits of *Amomum subulatum* were extracted with ethanol. A Silica gel TLC plates eluted with chloroform:methanol gave a chromatographically pure component – compound I. The structure of this compound has been elucidated via its spectral data. The aqueous extract of *Amomum subulatum* fruits was assessed for antimicrobial activity against five standard bacteria. The extract showed significant activity against the bacterial strains: *Pseudomonas aeruginosa* and *Bacillus subtilis*. It also showed good anticandidal activity.

1-Introduction

Amomum subulatum **Roxb.** has been used since ancient times as spice. It is a herbaceous crop widely cultivated for its economic value. The genus *Amomum* which comprises about 150 species belong to the family Zingiberaceae¹.

Amomum subulatum is astringent, stomachic, alexipharmic and stimulant². The oil is used traditionally against indigestion, abdominal pain and rectal disorders³. The plant is also used for digestive troubles, inflammation of eyelids, congestion of lungs and pulmonary tuberculosis beside liver complaints and loss of appetite⁴.

A mixture of this herb and cuminum is used traditionally against malaria⁵. Seed decoction is used in ethnomedicine for gum infection and pericarp is a remedy for headache and stomatitis⁶. Seeds which are useful for snake bite are also used to inhibit hyperlipidaemia⁷. Seed decoction is reported to possess analgesic properties⁶.

While the aqueous and ethanolic extracts exhibited antiinflammatory effect⁸, Kumar⁹ demonstrated the antibacterial properties of *Amomum subulatum* extracts. The volatile oil from

this species showed significant antifungal activity¹⁰. The seeds which contain a among others – 1,8-ceineol- have free radical scavenging capacity¹¹.

2. Materials and Methods

2.1- Materials

2.1.1- Solvents

All solvents used are of analytical grade(Loba Chemicals-India). Methanol HPLC grade is used for spectroscopic purposes(Sigma-Aldrich , England).

2.1.2- Plant material

Fruits of *Amomum subulatum* were purchased from the local market- Khartoum (Sudan) . The plant was identified and authenticated by The Institute of Medicinal and Aromatic Plants, Khartoum, Sudan.

2.1.3- Equipments

Ultraviolet absorption spectra were obtained in spectroscopic methanol on UV -Visible Spectrophotometer(Shimadzu). When visualizing TLC plates a multiband UV λ_{max} (254 / 365 nm) portable ultraviolet lamp, a product of Hanovia lamps (6 watt S/Y and L/W) was used.. ¹HNMR spectrum was obtained on a Bruker AM 500 spectrophotometer (Germany) operating at 500 MHz in spectroscopic grade DMSO-d₆. The chemical shifts values were expressed in δ (ppm) units using (TMS) as an internal standard.

2.1.4-Test organisms

Standard microorganisms used for antimicrobial screening are depicted below:

Table 2.1: Test organisms

Ser. No	Micro organism	Type
1	<i>Bacillus subtilis</i>	G+ve
2	<i>Staphylococcus aureus</i>	G+ve
3	<i>Pseudomonas aeruginosa</i>	G-ve
4	<i>Escherichia coli</i>	G-ve
5	<i>Candida albicans</i>	fungi

2.2- Methods

2.2.1- Extraction of flavonoids

Powdered shade-dried fruits *Amomum subulatum* (1Kg) were extracted with 95% ethanol for 72 hours. The solution was filtered and evaporated to dryness under reduced pressure.

2.2.2-Isolation of flavonoids

The crude ethanolic extract was applied on TLC plates (20x20cm) as narrow strips. The bands were eluted with 15% acetic acid. The developed chromatograms were air-dried and examined under both visible and UV light (λ_{\max} 366,245nm).. The equivalent bands from each plate were then scratched, combined and slurred with methanol. After several hours of contact, with occasional shaking, the liquid was evaporated *in vacuo* to dryness. In this way compound I (R_f 0.70) was obtained in a chromatographically pure form.

2.2.3-Antimicrobial test

The aqueous extract of *Amomum subulatum* fruits was screened for its antimicrobial activity against five standard human pathogenic bacterial strains: Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungal species : *Candida albicans*. The cup plate agar diffusion bioassay was used.

2.2.3.1- Preparation of bacterial suspensions

One ml aliquots of a 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 100 ml sterile normal saline solution 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.

2.2.3.2- 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.

2.2.3.3- Antibacterial susceptibility

The cup plate agar diffusion assay was used to screen the antibacterial activity of the sample and performed by using Mueller Hinton agar (MHA).

(2ml) of the standardized bacterial stock suspension were mixed with (200 ml) of sterile molten nutrient agar which was maintained at 45°C. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle. Each plate was divided into two halves. In each half two cups (10mm in diameter) were cut using sterile cork borer (No 4). Each half was designed for a test solution.

Agar discs were removed, alternate cups were filled with(0.1 ml) samples of each test solution and allowed to diffuse at room temperature for two hours. The plates were then incubated at 37°C for 24 hours. After incubation, the diameters of the resultant growth inhibition zones were measured in duplicates and averaged.

2.2.3.4-Antifungal susceptibility

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

3-Results and Discussion

3.1-Flavonoids of *Amomum subulatum*

The fruits of *Amomum subulatum* were macerated with 95% ethanol for 72hr. The solvent was removed *in vacuo* giving a crude product. A Silica gel TLC plates eluted with chloroform: methanol (3:7; v:v) gave a chromatographically pure component – compound I. The structure of this compound has been elucidated via its spectral data(UV and NMR).

3.2- Compound I

Usually when considering structural elucidation of flavonoids, the first step is to conduct extensive UV studies ,including the use of UV shift reagents, to know the class to which the flavonoid belongs.Flavonoids usually exhibit two absorption bands ; band I and II. Band I is

due to the absorption of the cinnamoyl system, while band II originates from the benzoyl system.

It is known that flavones, flavonols, chalcones and aurones give both band I and II, due to effective conjugation between the carbonyl function and the aromatic B ring. Flavanones, isoflavones, dihydroflavonols and dihydrochalcones give only band II in the range : 230-290nm. These classes of flavonoids are known to lack conjugation between the B ring and the carbonyl function and thus afford absorption due to the benzoyl chromophore only.

In the UV compound I absorbs (Fig.1) at λ_{max} 234nm. Such absorption is characteristic of :flavanones, isoflavones, dihydroflavonols and dihydrochalcones.

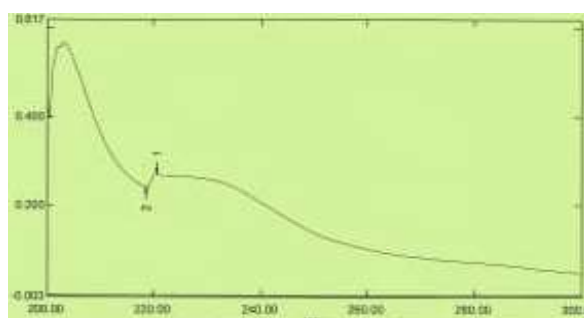
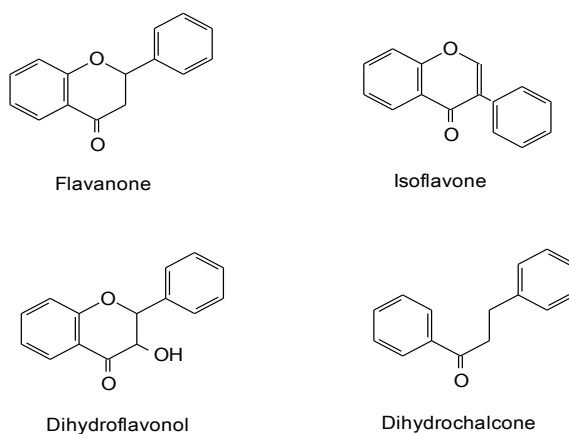


Fig.1 :UV spectrum of compound I



Among the flavonoids , isoflavones possess a spectrum characterized by a shoulder in the range 400-430nm and such shoulder was not detected in the UV spectrum(Fig.1) of compound I . Dihydroflavonols are distinguished by a 3-OH function which is detectable by the shift reagent –sodium methoxide. This reagent also detects a 4`-OH function. In both cases the sodium methoxide spectrum shows a bathochromic shift accompanied by a decrease in intensity in case of a 3-OH function.

However, the sodium methoxide spectrum(Fig.2) gave λ_{\max} 207nm i.e. it did not show any bathochromic shift indicating absence of 3- and 4` -OH and consequently absence of dihydroflavonols. Such argument suggests that the isolated flavonoid is either a flavanone or a dihydrochalcone.

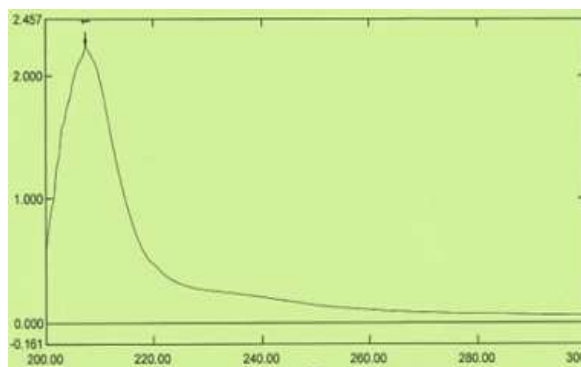


Fig.2 :Sodium methoxide spectrum of compound I

Due to the mutual splitting of the unequivalent C-3 protons in flavanones a double doublet appears around δ 2.8ppm. Such doublets are further split into a multiplet by C-2 protons. Another multiplet arising from C-2 protons being split by C-3 protons appears around δ 5.2ppm. Thus the ^1H NMR can distinguish between flavanones and dihydrochalcones. The ^1H NMR of compound I(Fig.3) did not reveal such multiplets indicating that the isolated compound is a dihydrochalcone.

The ^1H NMR spectrum(Fig.3) showed : δ 1.22(3H) which was assigned for a methyl group. The signal at δ 1.73ppm which integrates for 6 protons accounts for two acetyl functions, while the resonance at δ 4.30(6H) ppm is due to two methoxyl groups. The resonance at δ 6.62 ppm accounts for the aromatic protons .

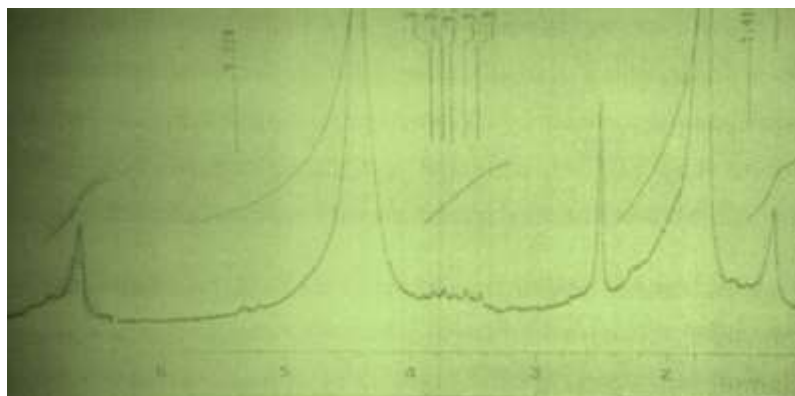


Fig. 3 : ^1H NMR spectrum of compound I

Sodium acetate is a useful UV shift reagent used for the specific detection of a 7-OH group. In presence of a 7-OH function, the sodium acetate spectrum affords a bathochromic shift. However this spectrum did not afford any bathochromic shift indicating absence of a 7-OH function (Fig. 4).

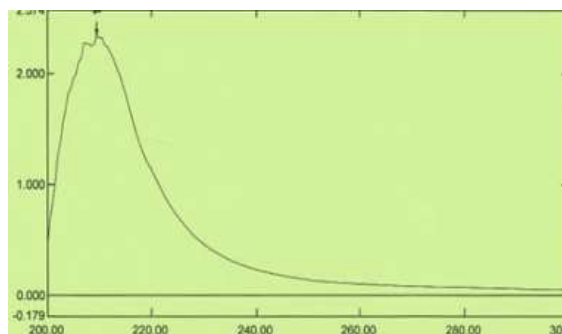
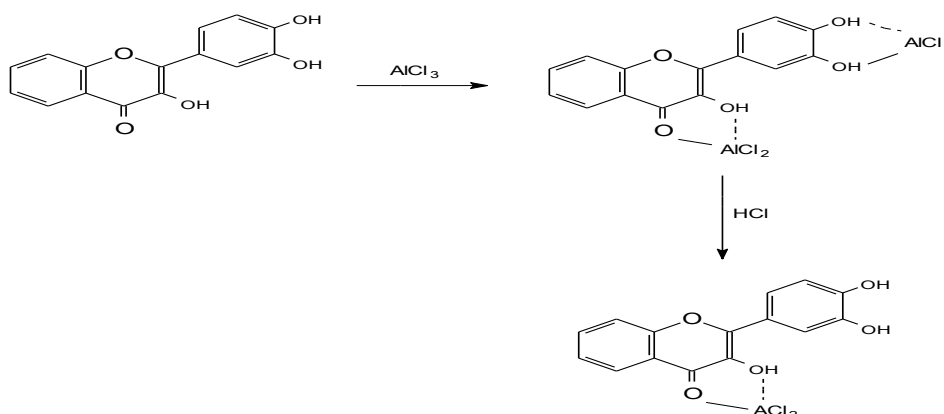


Fig.4 : Sodium acetate spectrum of compound I

Aluminium chloride is another useful UV shift reagent capable of detecting catechol systems as well as 3- and 5-OH functions in the nucleus of flavonoids. This reagent can form complexes with a 3-OH and a 4-keto function; a 5-OH and a 4-keto group. It can also chelates with catechols systems in both aromatic rings. Though the catechol complexes decompose in acidic media, the 3- and 5-OH complexes are quite stable in such media. Such complexes are displayed below:



The aluminium chloride spectrum (Fig. 5) did not give a bathochromic shift indicating absence of 5- and 3-OH functions as well as catechol systems. The boric acid spectrum (Fig.6) which is diagnostic of catechol systems did not reveal any bathochromic shift indicative of such catechols.

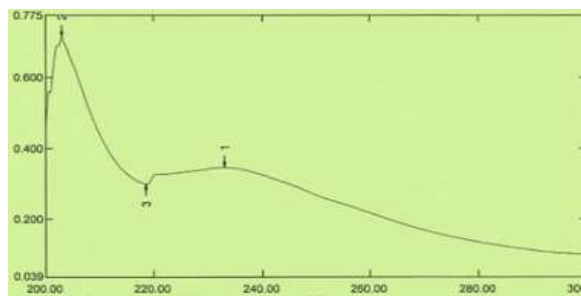


Fig.5 : Aluminium chloride spectrum of compound I

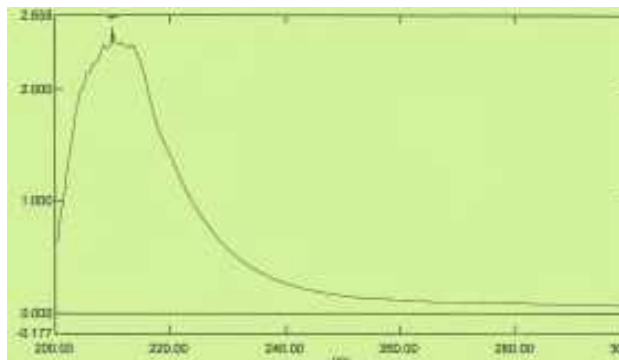
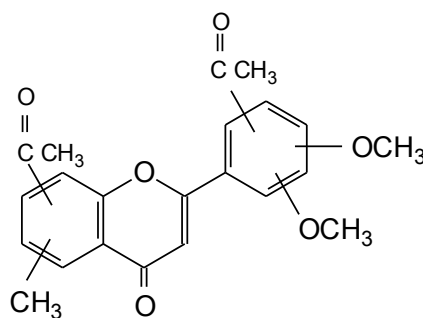


Fig.6 : Boric acid spectrum of compound I

On the basis of the above spectral data the following partial structure was assigned for compound I:



Compound I

3.1.2-Antimicrobial activity

The aqueous extract of *Amomum subulatum* fruits was assessed for antimicrobial activity against five standard bacteria. It showed significant activity against the bacterial strains : *Pseudomonas aeruginosa* and *Bacillus subtilis* . It also showed good anticandidal activity (Table3.1).The antimicrobial activity of standard drugs are depicted in Tables 3.2 and 3.3.

Table 3.1: Antimicrobial activity of *Amomum subulatum* fruit extract

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca
<i>Amomum subulatum</i>	100					
Ethanollic extract	100	15	20	10	20	15

Table 3.2 : Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

Drug	Conc.mg/ml	Bs.	Sa.	Ec.	Ps.
Ampicillin	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 3.3 : Antifungal activity of standard chemotherapeutic agent

Drug	Conc.mg/ml	Ca.
Clotrimazole	30	38
	15	31
	7.5	29

Sa.: *Staphylococcus aureus*

Ec.: *Escherichia coli*

Pa.: *Pseudomonas aeruginosa*

Ca.: *Candida albicans*.

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