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Isolation and partial structure of a flavone from *Combretum aculeatum* (Combretacea) leaves and antimicrobial activity of some fractions

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Abstract
From ethyl acetate extract of the leaves of *Combretum aculeatum* a flavone was isolated via column and paper chromatography. The structure of the flavone was partially elucidated by a combination of spectral techniques (UV, IR, ^{1}HNMR and MS). The methanolic and ethyl acetate fractions were evaluated for their antimicrobial activity. The methanolic extract showed significant activity against *Escherichia coli* and *Pseudomonas aeruginosa*. It also exhibited moderate activity against *Bacillus subtilis* and *Staphylococcus aureus*. This extract also exhibited significant antifungal activity against the fungi: *Candida albicans and Aspergillus niger*. However, the ethyl acetate extract was devoid of activity.

Keywords: *Combretum aculeatum,* isolation, partial structure, flavone, antimicrobial activty

Introduction

Combretum aculeatum – known locally as "Sheheit"- is a plant in the family Combretaceae. This species is a common plant in Sudan. In Africa it is stretching from Senegal and Mauritania, to Somalia and Tanzania. *Combretum aculeatum* is a climbing shrub growing up to 4 m, even taller if support is available [1]. The plant is an important nutrient for animals, which consume the leaves, flowers and young shoots [1, 2]. *Combretum aculeatum* is used traditionally as purgative and diuretic [1, 3]. Pharmacological studies of leaves and roots extracts showed antibacterial activity [4]. Phytochemical screening revealed the presence of flavonoids among others.

Flavonoids are a group of natural compounds with variable structures. They are found in fruits, vegetables, grains, barks, roots, stems, flowers and tea [5, 6]. The chemical structure of these phenolics is based on a C_{15} skeleton consisting of two aromatic rings (A) and (B) linked by a heterocyclic ring (C). The (C) ring is sometimes replaced by a five-membered ring giving a class of flavonoids known as aurones [6].

Depending on the saturation of the (C) ring and position the aromatic (B) ring, flavonoids can be subdivided into different subgroups or classes [5]. The subgroups include: flavones, flavonols, flavanones, flavanonols, anthocyanins, isoflavonoids, chalcones and aurones [5, 6].

Flavonoids exhibit diverse biological activities including antioxidant, anti-inflammatory, antiallergic, antischemic, antiplatelet, immunomodulatory, and antitumor activity [7-12].

Materials and Methods

Materials

Analytical grade reagents were used. The UV spectra were recorded on a Shimaadzu UV – 2401PC Spectrophotometer and UV lamp was used for localization of spots on TLC plates. Nuclear Magnetic Resonance spectra were run on a Joel ECA 500MHZ NMR Spectrophotometer. Mass spectra were measured on a Joel MS Spectrometer (JMS-AX500).

Plant Material

The leaves of *Combretum aculeatum* were collected form Basunga, Gadaref State-Sudan. The plant was authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum.

Methods

Extraction and Isolation of Flavonoids

Powdered air-dried leaves of *Combretum aculeatum* (1Kg) were macerated with 95% ethanol for 72h 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° C until all methanol was removed yielding a crude product, which was suspended in 300 ml water and left overnight in a refrigerator and then filtrated. The aqueous filtrate (500 ml.) was partitioned successively with n-hexane, dichloromethane, ethyl acetate and butanol.

Column Chromatograpy

Open column (80 x4 cm) was used for fractionation the ethyl acetate fraction. Silica gel with particle size 100-200 mesh from LOBA chemicals (India) was used as stationary phase. The composition of the mobile phase (dichloromethane CH_2Cl_2: methanol CH_4OH) was determined by TLC analysis.

The column was packed with slurry of silica gel with dichloromethane and then allowed to equilibrate for two hours before use.

The ethyl acetate fraction 5 g was mixed with 10 g of silica

gel and then applied on the top of the column. Elution commenced by CH_2Cl_2: MeOH 95:5 in increasing order of polarity stepwise until 100% methanol. Fractions of 10 ml were collected, and concentrated under reduced pressure then investigated by thin layer chromatography (TLC) analysis using different solvent systems.

The spots were visualized under UV lights using both short and long wavelengths with and without exposure to NH_3 and sprayed with NA reagent. Similar fractions were combined and concentrated to dryness.

Preparative Paper Chromatography

Preparative paper chromatography was carried out for further purification using BAW (Butanol: Acetic acid: Water 4:1:5 upper layer) as mobile phase. The developed chromatograms were dried, sprayed with NA reagent and the colours (bands) which developed were observed in UV light (λ = 366, 254 nm) and determined. The equivalent bands from each paper were then cut out into small strips and slurred with methanol. After several hours of contact with occasional shaking, the liquid was filtrated and evaporated to dryness. In this way compound I was isolated.

Antibacterial Activity

Using the cup plate agar diffusion bioassay [13], the antimicrobial activity of the methanol and ethyl acetate fractions was assessed against the pathogenic bacteria (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger*).

Results and Discussion

The partial structure of compound I, which was isolated as yellow powder from *Comretum aculeatum* leaves was elucidated via a combination of spectral tools (UV, ^{1}HNMR and MS).

In the UV, compound I absorbs at λ_{max} 271,328nm (Fig. 1). This absorption is characteristic of flavones [13, 14]. When the shift reagent, sodium methoxide, was added to a methanolic solution of compound IV a bathochromic shift-without decrease in intensity- was observed indicating [13] a 4`-OH function (Fig. 2).

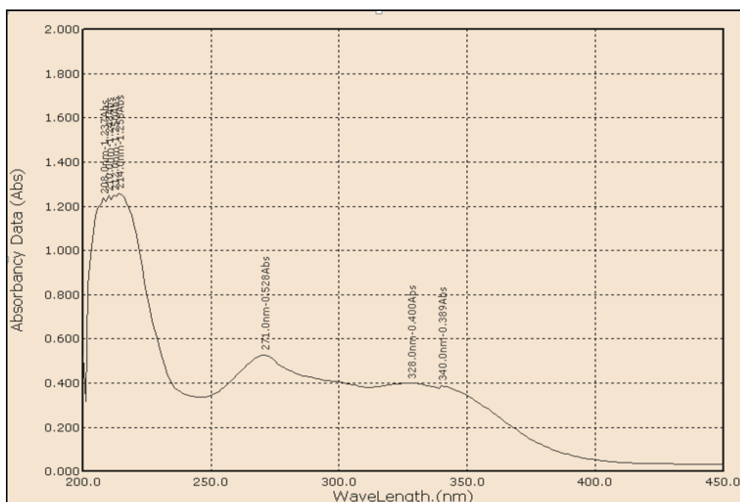


Fig 1: UV spectrum of compound I

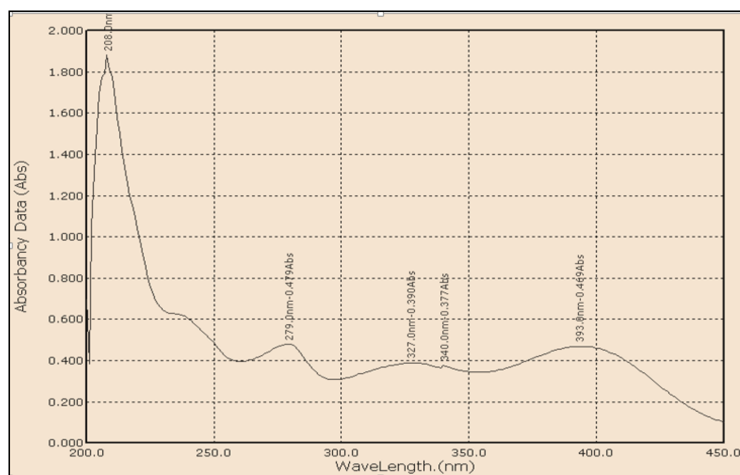


Fig 2: Sodium methoxide spectrum of compound I

The sodium acetate spectrum (Fig.3) revealed a bathochromic shift which is diagnostic [13, 14] of a 7-OH.

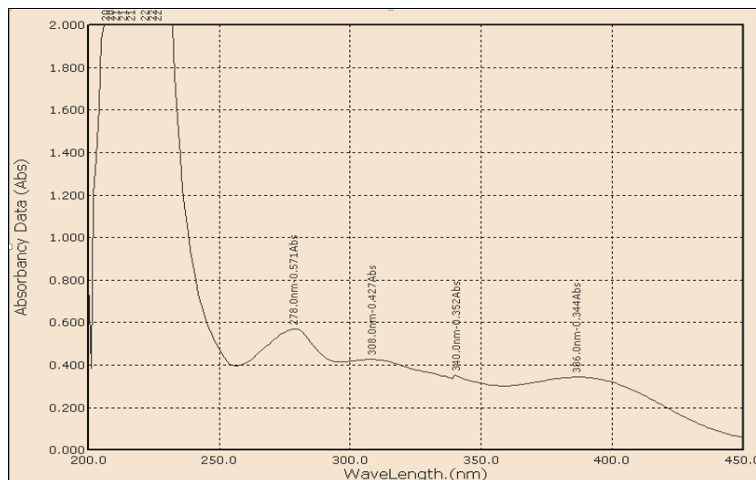


Fig 3: Sodium acetate spectrum of compound I

No bathochromic shift was observed in the aluminium chloride spectrum and this indicates [14] absence of catechol systems as well as a 5-OH function (Fig. 4). The same trend was observed in the boric acid spectrum which is diagnostic [13, 14] of catechol systems (Fig. 5).

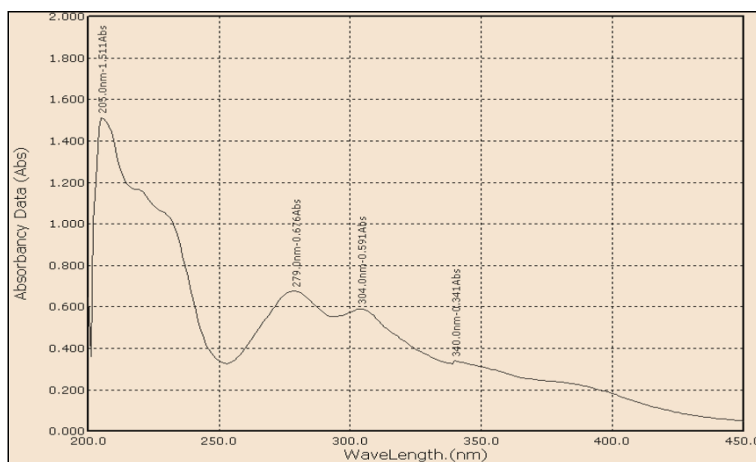


Fig 4: Aluminium chloride spectrum of compound I

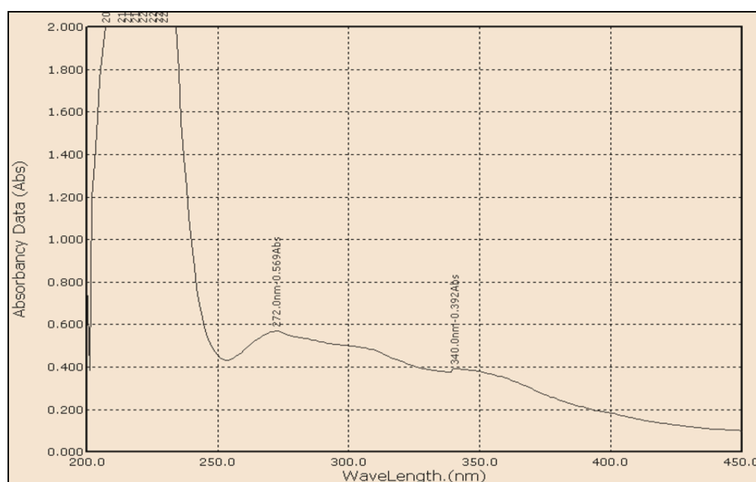


Fig 5: Boric acid spectrum of compound I

The ^{1}HNMR spectrum (Fig. 6) showed a signal at δ0.84 assigned for a methyl group. The resonance at δ3.91 accounts [13] for a methoxyl function, while The double doublet at δ6.70 and δ6.93 is characteristic of C_6- and C_8- protons respectively.

The low field signal at δ8.05 was attributed to C_5-H.This proton resonates at lower field relative to other (A) ring protons due to the deshielding effect of the neighboring 4-keto function. The resonances at δ7.69 and δ 7.86 ppm account for the B ring protons. The mass spectrum gave m/z300 for $M^+ + 2H$.

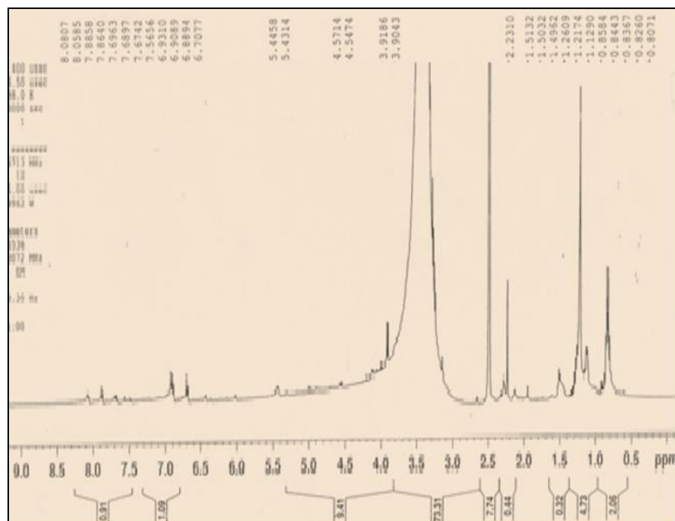
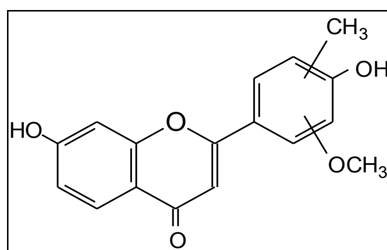


Fig 6: ^{1}HNMR spectrum of compound IV

Thus the following partial structure was assigned for this flavone:



Compound I

Antimicrobial Assay

The methanol and ethyl acetate extracts of *Combretum aculeatum* were evaluated for their antimicrobial potential against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*). The methanol extracts showed significant activity against *Escherichia coli* and *Pseudomonas aeruginosa*. It also showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus.* This extract also exhibited significant antifungal activity against the fungi: *Candida albicans and Aspergillus niger*. However, the ethyl acetate extract was devoid of activity. Inhibition zones are depicted in Table 1. Tables 2 and 3 display the antifungal and antibacterial activities of standard chemotherapeutic agents respectively.

Table 1: Antimicrobial activity of *Cumbretum aculeatum* fractions

Extract	Con.(mg/ml)	Sa	Bs	Ec	Pa	Ca	An
Methanol	100	17	17	22	30	19	22
Ethyl acetate	--	--	--	--	--	--	--

Table 2: Antifungal activity of standard chemotherapeutic agent

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

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

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

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