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Phytochemical Screening, Characterization and Antimicrobial Activity of a Flavonoid from Sudanese *Bauhinia rufescens* (kulkul) (Caesalpiniaceae) Roots

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Authors' contributions

This work was carried out in collaboration between both authors. Author NKH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SAG managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

A 7-, 4′ dihydroxyl-5-methoxyl -5′ acetyl flavonoid was isolated from the roots of Sudanese *Bauhinia rufescens* (family, Caesalpiniaceae Fabaceae). The isolate was purified by different Chromatographic techniques and identified via a combination of spectral tools (IR, UV, ^{1}HNMR and Mass spectroscopy).

The isolated compound was screened for its antimicrobial activity against six standard human pathogens (*Staphylococcus aureus*, *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger* and *Candida albicans*) and promising results were obtained. About 600 species of *Bauhinia* grow in the tropical regions of the word. It is found in the Sahel and adjacent Sudan zone, from Senegal and Mauritania across North Ghana and Niger to central Sudan and Ethiopia. *Bauhinia rufescens* is a little tree shrub. green all year with small leaves and white flowers. The macerate of *Bauhinia rufescens* roots used externally to treat ulcers and rheumatism. The isolated flavanone can play role in development of new tool as antimicrobial agent.

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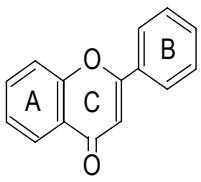
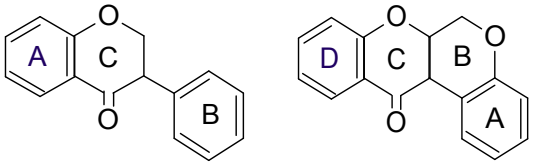
1. INTRODUCTION

Flavonoids are a group of polyphenolic compounds that are widely distributed in the plant kingdom. They occur naturally as plant pigments in a broad range of fruits and vegetables as well as beverages [1], flavonoids are phenolics comprising 15 carbons, with two aromatic rings bound together by three carbon atoms that form an oxygenated heterocyclic rings(C_6-C_3-C_6). They are found throughout the plant kingdom and in particular in leaves and in the skin of fruits. Based on the variation of their heterocyclic ring, flavonoids are divided into different sub-classes: Flavones, flavonols, flavans, flavanones, flavanols, chalcones, dihydrochalcones, isoflavones,neurons, anthocyanins, anthocyanidins,and catechins. The basic C_6-C_3-C_6 flavonoid skeleton can have numerous substituents (e.g. hydroxyl, acetyl, methoxyl and methyl groups) and the majority of the flavonoids exist naturally as glycosides [2,3]. Flavonoids have gained recent interest because of their broad biological and pharmacological activities. Flavonoids have been reported to exert multiple biological effects including antimicrobial [4], cytotoxicity [5], anti-inflammatory [6], as well as antitumor activities [7,8]. Flavonoids exhibit anti-oxidant, immune modulatory, chemopreventive and anticancer properties [9]. Flavones are the root from which the word flavonoid is derived. They are also known as anthoxanthins. They are yellow pigments which are widespread in leaves and flowers of angiosperm [10]. Flavones can be found in all parts of plant, above and below ground, in vegetable and generative organs. They are in stems, leaves, buds, barks, heartwood, thorns, roots, rhizomes, flowers and also in root and leaf exudates or resins. Flavones producing plant species belong to more than 70 different families within the plant kingdom representing an abundant class of phytochemicals in our daily diet. Lastly, flavones attracted considerable scientific and therapeutic interest, because of the assumed beneficial health effects of flavones in the prevention of some human diseases [11].



The isoflavones form one of the larger classes of natural products. The relationship of their structure to the skeleton of the rotenoids structure as shown below.



Isoflavone occur naturally, but are not as widespread as the flavones [10]. They are occurring either in the free state or as glycosides. Glycosides of isoflavones have been known since a very early date. Natural glycosides, however, have been reported, mainly as a result of more systematic analysis of plant extractives . The majority of glycosides are (7-glucosides) or (7- rhamnosyl-glucoside) and (4- glucosides) or (4-rhamnosyl glycosides) [12]. Isoflavones are difficult to characterize since they do not respond specifically to any one color reaction. Some isoflavones give a light blue colour in UV- light in the presence of ammonia, but most other appear as dull- purple absorbing spot, changing to dull-brown with ammonia. In fact in some early investigations, isoflavones have been mistaken for flavones owing to their similar behavior in certain colour tests. Various color reaction may be used to test for benzopyrone structure, but they do not apparently differentiate between flavones and isoflavones [13]. Flavonoids with a hydroxyl functions at C4′ and C_7 have shown significant cytotoxic effects against tumor cells, compared with other structurally related flavonoids. The hydroxylation at C6 plays an important role in antioxidant activity of flavonoids [14].

About 600 species of *Bauhinia* grow in the tropical regions of the world [15]. The genus includes tree, vines, and shrubs that are frequently planted for their showy flowers and ornamental foliage. A native of southeastern Asia, *B. monandra* is found in the tropical regions of the world. Cultivated in the West Indies, it has naturalized throughout the islands [16]. *Bauhinia rufescens* is deciduous in drier areas and is an evergreen in wetter areas. It is often found in dry savannah, especially near stream banks. It is found in the other Sahel and adjacent Sudan zone, from Senegal and Mauritania across North

Ghana and Niger to central Sudan and Ethiopia [17]. This is a beautiful little tree shrub. It stays green all year round and has small leaves like lima beans and little white flowers like confederate jasmine. *Bauhinia* used externally to treat ulcers and rheumatism; these and several other medicinal uses. Today, the plant is still sometimes used for these purposes in folk medicine [18].

The objective of this study was to evaluate the antimicrobial activity of *Bauhinia rufescens* roots. *Bauhinia rufescens* belongs to family

Caesalpiniaceae, yet an important medicinal plant of Sudan

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and instruments

The UV spectra were recorded on a Shimadzu 1601 Spectrophotometer and UV lamp was used for localization of fluorescent spots on TLC and PC. The IR spectrum was recorded as KBr disk , using Perkin-Elmer, FTIR 1600-Jasco. Nuclear Resonance Spectrum was run on a Bruker AM 500 MHZ NMR Spectrophotometer. The mass spectrum was recorded by direct probe EIMS using a Shimadzu QP-class-500 spectrometer.

2.2 Plant Material

The roots of *Bauhinia rufescens* were collected in May 2014 from South Kordofan -Sudan. The plant was identified and kindly authenticated by the Department of Botany, University of Khartoum.

2.3 Extraction of Plant Constituents

Powdered air-dried roots of *Bauhinia rufescens* (1kg) were exhaustively macerated with 95% ethanol at room temperature for 48hr. The solvent was removed under reduced pressure to afford a solid. The crude extract was suspended in water and partitioned with organic solvents in order of increasing polarity: chloroform, n-butanol, ethyl acetate and petroleum ether.

2.4 Isolation of Flavonoid

Then- butanol fraction was rich in phenolics. It was dissolved in methanol and applied on Whatman paper (No 3mm-46x57cm). The bands were sprayed 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= 366,245 nm). The chromatograms were exposed to ammonia vapor for about 2-3 seconds and immediately re-examined to observe possible changes that may eventually appear in color or fluorescence under a long wavelength UV lamp. 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 filtered and evaporated under reduced pressure to ensure compound I.

2.5 Preliminary Phytochemical Screening

The ethanolic extract of *Bauhinia rufescens* roots was screened for steroids, flavonoids, alkaloids, glycosides, tannins and saponins according to the method described by Harborne (2001).

2.6 Anti-microbial Assay

The isolated flavonoid was screened for its antimicrobial activity against four bacterial strains: Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa and Escherichia coli*) and two fungal species (*Aspergillus niger* and *Condida albicans*). The cup plate agar diffusion method was used.

2.7 Preparation of Bacterial Suspensions

One ml aliquots of 24 hours broth cultures of 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, to produce a suspension containing about 10^8 -10^9 colony forming units per ml. The average number of the viable organism per ml of saline suspension was determined using surface counting technique. The suspension was stored in the refrigerator at 4°C till used. Serial dilution of the stock suspension were made in sterile normal saline in tubes and adjustable volumes micropipette transferred one drop (0.02 ml) volumes of the appropriate dilution onto the surface of dried 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^{o}C for 24 hours. After incubation, the number of developed

colonies in each slide 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 colonies forming units per ml suspension. Each time new stock suspension was prepared. All the above experimental condition were maintained constant so that suspension with very close viable counts would be obtained.

2.8 Preparation of Fungal Suspensions

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

2.9 Testing for Antibacterial Activity

The cup –plate agar diffusion method was adopted with some minor modification, to assess the antibacterial activity. 20 ml aliquots of incubated agar were distributed into sterile Petri-dishes, the agar was left to set in each of these plates which were divided into two halves cup in each half (10 mm in diameter) were cut using a sterile cork- borer (No 4). Each of the halves was designed for one of the test compounds. Separate Petri- dishes were created for the standard antibacterial chemotherapeutic agent. The agar discs were removed, Alternated cups were filled with 0.1 ml sample of each of the extracts and pure compounds using adjustable volume micro titer pipette, and allowed to diffuse at room temperature for two hours. The plats were then incubated. In the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentration of the isolated compounds and the standard antibacterial chemotherapeutic. After incubation, the diameter of the resultant growth inhibition zones was measured and averaged.

2.10 Testing for Antifungal Activity

The method as mentioned above was adopted for Antifungal Activity, but instead of nutrient agar saturated dextrose agar was used. Samples were used here by the same concentration used above.

3. RESULTS AND DISCUSSION

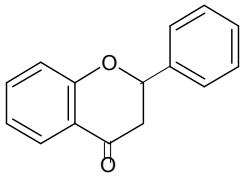
3.1 IR spectrum

The IR spectrum of compound I showed υ (KBr,cm^{-1}): 3412.50 (OH) , 2896.8 (C-H ,aliph.), 1650.81(C = O), 1450.90 (C = C, aromatic), 1163.0 (C-O, ether), and 1060.91 (CO, phenolic). Since the IR revealed a C = O function, hence compound I cannot be an anthocyanin or catechin.

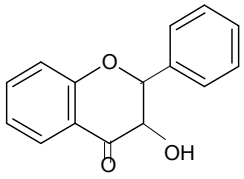
3.2 UV Spectrum

The UV spectrum (Fig.2) showed λmax (MeOH275 nm. Since compound I gave the only band II it could be (i) a flavone (ii) isoflavone (iii) dihydoflavanol or (iv) dihydrochalcone.

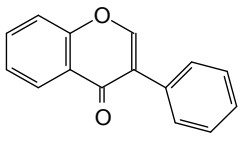
The UV spectrum did not reveal any shoulder characteristic of isoflavones(19), in the range: 300-340 nm.



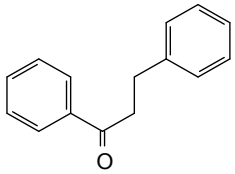
Flavanone



Dihydroflavono



Isoflavone



Dihydrochalcone

3.3 Sodium methoxide spectrum

The sodium methoxide spectrum (Fig. 3) revealed a bathochromic shift without a decrease in intensity diagnostic of 4′ OH function [19]. Such UV data suggest a flavonoid.

3.4 Sodium Acetate Spectrum

When sodium acetate added to a methanolic solution of the compound, I, a 25 nm bathochromic shift was observed indicating the presence of a 7 –OH function (Fig. 4).

3.5 Boric acid and Aluminum Chloride Spectrum

No detectable bathochromic shift was observed in the boric acid spectrum (Fig. 5). Also, the AlCl3 range (Fig. 6) did not reveal any bathochromic shift indicating the absence of 3-,5-OH groups and catechol moieties [20].

Thus the private flavanone lakes 3-,5-, hydroxylation and catechol systems as indicated by various shift reagents.

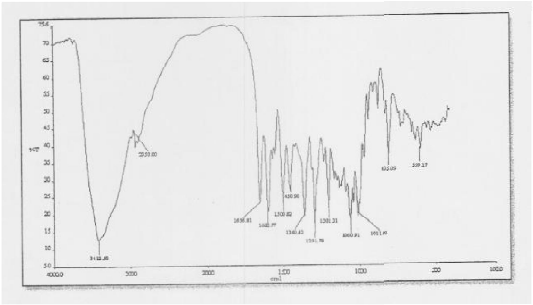


Fig. 1. The IR spectrum of compound I

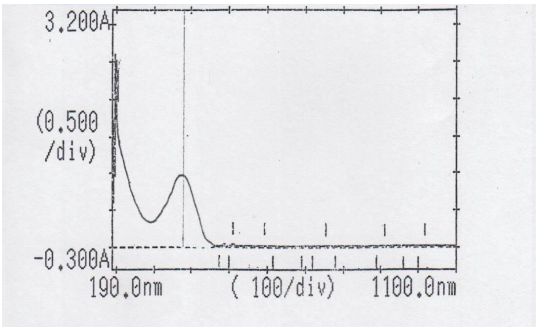


Fig. 2. UV spectrum of compound I

3.6 HNMR Spectrum

The ^{1}HNMR range (Fig. 7) showed a signalled at δ 2.4 for an acetyl function; δ 3.6 assigned for methoxide function. The resonance at δ 8.5 was awarded for the aromatic proton.

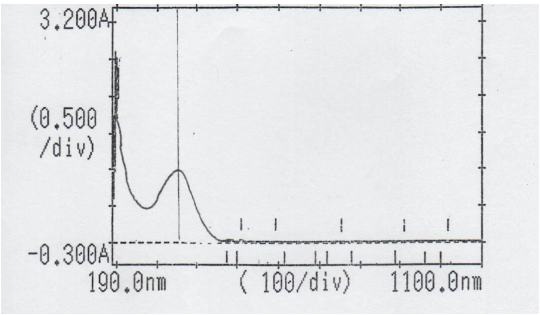


Fig. 3. Sodium methoxide spectrum of compound I

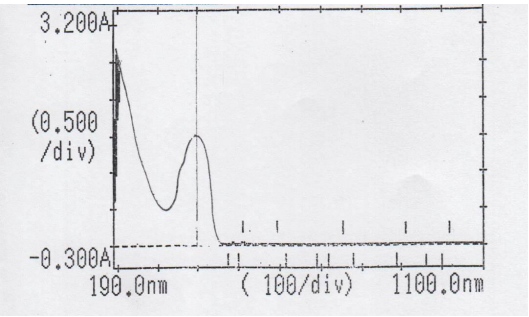


Fig. 4. Sodium acetate spectrum of compound

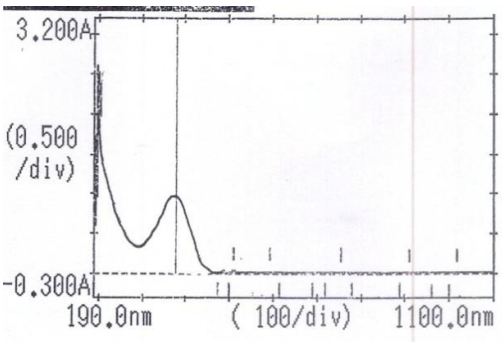


Fig. 5. Boric acid spectrum of compound I

3.7 Mass Range

The mass spectrum (Fig.8) gave m\ z 329 (M+) for the aglycone. Other vital fragments corresponding to intact aromatic rings were shown at m\z 167(ring A) and m\z162(ring B). H′-H′ COSY NMR experiments indicated a long-range coupling between the acetyl protons and C_{6}-H. Hence the acetyl function is substituted at $C_{5'}$ of B-ring. The citation methoxyl group at position 5 of rang A was based on H′-H′ COSY NMR experiments which indicated the long-range

coupling between the methoxyl function and C_6 – H.

3.8 Anti-microbial Activity

The mixture, I and fraction from *Bauhinia rufescens* (ethanol), were evaluated for potential antimicrobial activity against six standard human pathogens.

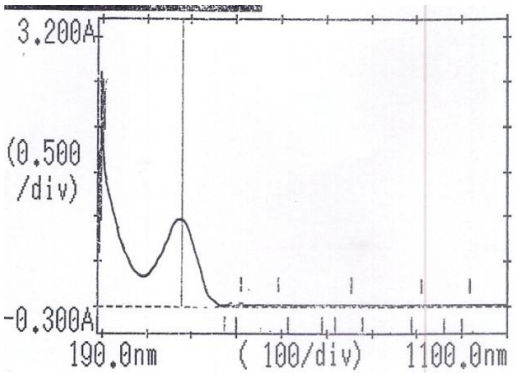
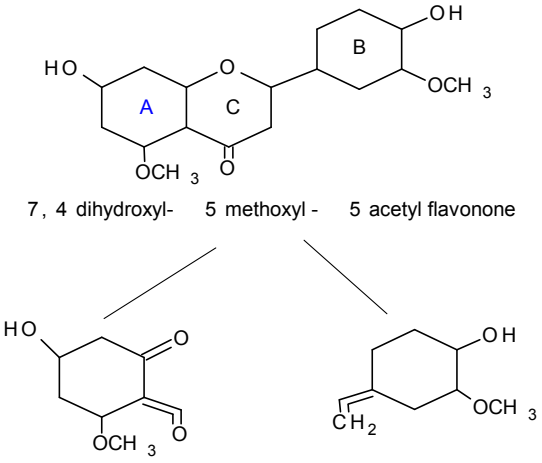


Fig. 6. Aluminium chloride spectrum of compound I

The average of diameters of the growth inhibition zones is shown in the table (1). The results were interpreted in a commonly used term: (<) 9 mm: inactive; 9-12 mm: partially active; 13-18 m: active ;(>) 18 mm: very intense.

Comparison with available literature data gave the following structure for compound I:



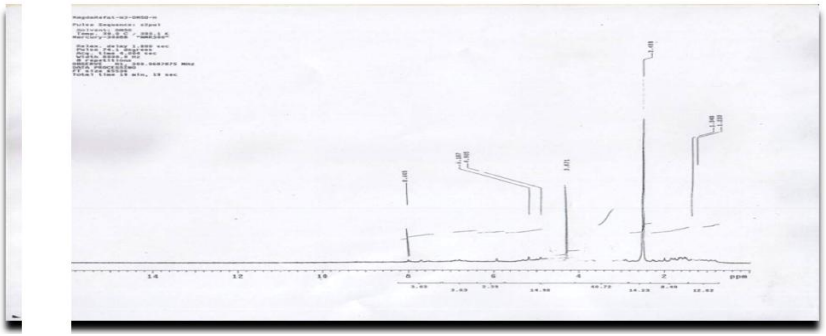


Fig. 7. The ^{1}HNMR spectrum of compound

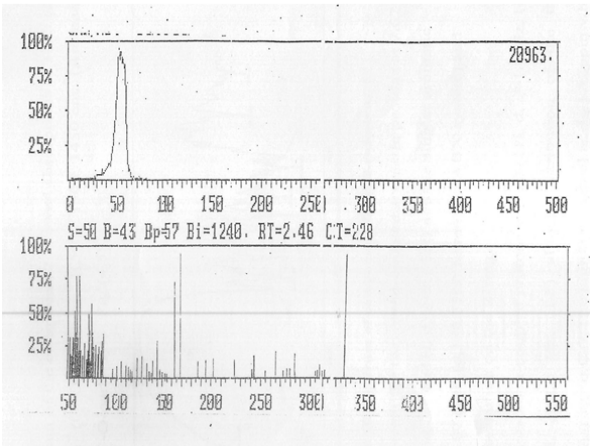


Fig. 8. The mass spectrum of compound I

Table 1. The Anti-microbial activity of compound I and *Bauhinia rufescens* ethanolic extract

Microorganism	Gram	Ethanolic fraction	Compound I(100mg\ml)
Staphylococcus aureus	+ve	22	20
Bacillus subtilis	+ve	21	17
Pseudomonas aeruginosa	-ve	24	20
Escherichia coli	-ve	26	20
Aspergillus niger	Fungus	18	14
Condida albacans	Fungus	16	16

4. CONCLUSION

A detailed phytochemical study of *Bauhinia rufescens* was carried out.From the ethanolic extract of *Bauhinia rufescens*, a flavonoid was isolated by chromatographic techniques. The isolate was identified via spectroscopic tools: IR, UV, 1HNMR and MS spectroscopy. The isolated compound exhibited promising activity against six standard human pathogens: *Staphylococcus aureus*, *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger* and *Condida albicans*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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