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Isolation and Structure Elucidation of Flavonoid from Leaves Extract of *Balanites aegyptiaca delil*

Tesfay Kahsay Gidey

Department of Natural Science and Mathematics, Adwa College of Teacher Education,Adwa, Ethiopia.

E-mail: tesfayk5@gmail.com

Abstract: Leaves of *Balanites aegyptiaca* have been used in Africa as folk remedy in the form of juice to treat diarrhea, dysentery, cuts and wounds. So this study is designed to characterize the isolated fraction and to explore the antioxidant and antibacterial activity of *Balanites aegyptiaca* leaves. In this work, a phytochemical study of *Balanites aegyptiaca* has been under taken and chemical investigation on the solvent extract of the plant has been conducted. Qualitative phytochemical analysis of methanol extract of *Balanites aegyptiaca* leaves showed the presence of saponin, alkaloids, phytosterols, resins, phenols, tannins, terpenes, flavonoids, proteins, and amino acids. The methanolic extract of leaves of *Balanites aegyptiaca* after repeated chromatography led to isolation of one compound, **BA-3** has been reported for the first time from this plant. The structure was elucidated from physicochemical characters, ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and HMQC and IR spectra data and by comparison of the data obtained with those reported for the compounds in the literature. Its complete structural elucidation requires the use of more efficient analytical techniques like high performance liquid chromatography (HPLC) and mass spectroscopy (MS).

INTRODUCTION

Balanites aegyptiaca is a multibranched, spiny shrub or tree up to l0m high [1,2]. Crown rounded, dense (but still seen through) with long stout branchlets. *Balanites aegyptiaca* is a species of tree, classified as a member of either as a member of Zygophylaceace or the balaniaceae comprising 9 species and 11 intraspecific taxa [3].

Many parts of the plant are used as famine foods in Africa; the leaves are eaten raw or cooked, the oily seed is boiled to make it less bitter and eaten mixed with sorghum, and the flowers can be eaten. The tree is considered valuable in arid regions because it produces fruit even in dry times. The fruit can be fermented for alcoholic beverages [2]. Seed oil contains the sapogenins-diosgenin and yamogenin [4,5]. Diosgenin can be used to produce hormones such as those in combined oral contraceptive pills and corticoids. The oil is used as cooking oil. The seed cake remaining after the oil is extracted is commonly used as animal fodder in Africa [3,6-8]. Almost all the parts of *Balanites aegyptiaca* plant are traditionally used in several folk medicines. In the Sahara region of Africa, the fruits are used as oral hypoglycemic drug [6].While the stem, root and leaf extracts of *Balanites aegyptiaca* have commonly been used as various traditional folk medicines especially in Africa and southern Asia [7-9]. The fruits are also commonly used as purgative, antiparasitic and schistosomicide [10-12]. *Balanites aegyptiaca* is one of the dominant tree species deliberately retained on farm fields in the Tigray region of Ethiopia. It is useful as a medicinal plant, food condiment, fodder, fuel wood, construction material and provision of shade. Although very little chemical investigation had been carried out on the *Balanites aegyptiaca*, series of studies on *Balanites aegyptiaca Delile* are currently under way. The higher oil content, its tolerance to drought and its unique character compared to other oil seed crops led me to study this plant.

MATERIALS AND METHODS

General Experimental Procedure

Sample leaf materials were air dried under shade for five days and the dried plant material was ground (January 2013) with a mortar and pestle. The groundedmaterial was stored in a refrigerator at 4 °C. IR spectra were recorded on Perkin-Elmer BX infrared spectrometer (400-4000 cm^{-1})using KBr. The ^{1}H, ^{13}C-NMR, DEPTand 2D NMR were obtained on a NMR machine (Bruker Avance 400 NMR spectrometer operating at 400 MHz). Both ^{1}H- and ^{13}C-NMR spectra were recorded in deuterated chloroform with small drop of deuterated methanol and the chemical shift values are expressed in parts per million (ppm) relative to the internal standard, tetramethylsilane. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; ddd, double double doublet; m, multiplet; br, broad and coupling constants (J values) are reported in Hertz (Hz). Thin layer chromatographic (TLC) analyses were performed on pre-coated silica gel 60 F_{254} plates (MERCK) and visualized by exposure to short wavelength UV (λ_{max}= 254 nm) and by spraying with 5 % methanolic NaOH spray reagent. All solvents used for chromatographic purposes were analytical grade except those for extraction processes, which were general purpose reagents (GPR).

Plant Sample Collection and Identification

Leaves of *Balanites aegyptiaca delile* were collected from Sefeho around the town of Axum in the Tigray People Region, 275 km north-western of Mekelle in January 2013. The plant was botanically authenticated in the Addis Abeba University. Voucher specimen was deposited at National Herbarium, Department of Biology, Addis Ababa University.

Preliminary Phytochemical Screening

The Soxhlet methanoland diethyl ether leaf extracts was analyzed for the presence of various phytoconstituents by following standard phytochemical tests and the results were reported. Preliminary phytochemical screening: It involves testing of different classes of organic compounds. The methods used for detection of different phytochemicals were followed by qualitative chemical test to give general idea regarding the nature of constituents present in the crude extract [13-16].

Isolation and Extraction of Leaves

Extraction for the phytochemical screening and phytochemical investigation was performed by Soxhlet extraction. Leaves were air dried under shade for five days

and the dried plant material was grounded (January 2013) with a mortar and pestle to obtain the powder of the crude extract. First, dried and powdered leaves were extracted with diethyl ether for four hours using Soxhlet extraction systems at room temperature and the extract was used for phytochemical screening. Then around 150g of the powder residue was packed in two thimbles of filter paper. The thimbles were then inserted into the Soxhlet apparatus and extraction was done by using 600 mL methanol as a solvent. The temperature was maintained at 70 °C and extraction was continued for 4 hours. Then the methanol extract was collected and powder from the thimble was discarded. Then the methanol extract was concentrated in rotary evaporator and dried in an oven at 45 °C, so as to obtain thick, viscous mass and yield value of extract was calculated. This dried extract was used for the phytochemical screening and characterization.

Fractionation, Isolation and Purification/ Chromatographic Technique

Isolation of Fraction-6

200 g of silica gel was measured and mixed with 400 mL of toluene and packed in to a column and 20 g of the thick waxy Soxhletextract of methanol after mixed with silica gel, it was subjected on top of the packed silica gel of column chromatography and then it was successively eluted with toluene: ethyl acetate: glacial acetic acid solvent system by increasing polarity. A total of 100 fractions were collected with10 mL. Fractions that showed the same R_f value and the same characteristic color on TLC were combined. Fraction (1 g) was subjected to repeated CC over silica gel (60 g, 60 X 2.5 cm)using petroleum ether: ethyl acetate solvent system and polarity was gradually increased by increasing the proportion of ethyl acetate. Thirty fractions (10 mL each) were collected and screened by TLC.

Table 1. Phytochemical screening for the extract of the leaves of *Balanites aegyptiaca*

S/N	Phytochemical test	Reagents used (test performed)	Result	
			ME	DEEE
1	Tests for alkaloids	Hager's Test	+	_
		Mayer's Test	+	_
2	Tests for flavonoids	Shinoda Test	+	+
		Lead Acetate Test	+	+
		Sodium Hydroxide Test	+	+
3	Test for Saponin	Foam Test	+	_
		Froth Test	+	_
4	Test for Phenolic comp.	Ferric Chloride Test	+	+
5	Test for tannins	Braemer's test	+	_
		Formaldehyde Test	+	_
		Gelatin Test	+	_
6	Test for terpenoids	Salkowski Test	+	_
7	Test for Anthraquinone glycoside	Borntrager's Test	+	_
8	Tests for Coumarins		_	_
9	Tests for Cardiac glycosides	Keller- Killiani Test	_	_
10	Test for steroids	Salkowski Test	_	_
		Libermann Burchard Test	+	_
11	Test for carbohydrates	Fehling's Test	_	_
12	Test for resins	Acetone-water test	+	+
13	Tests for proteins and amino acid	Biuret Test	+	_
		Xanthoproteic test	+	_
		Ninhydrin test	_	_
14	Test for Fixed Oils and Fats	Filter Paper /Spot Test	+	+

ME- Methanol extract **DEEE-** Diethyl ether extract

Fractionation of fraction was performed by flash chromatography over silica gel column,11 mm X 22 cm, using chloroform: ethyl acetate as eluent and polarity was gradually increased by increasing the proportion of ethyl acetate. Fifteen fractions (20 mL each) were collected and screened by TLC.

17 mg of fraction was collected and showed spot on TLC using UV lamp at 254 nm. It was then characterized after purification using spectroscopic techniques and identified.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The methanol extract of the leaves of *Balanites aegyptiaca* was subjected to preliminary phytochemical screening tests.

The positive (+) and negative (–) signs indicate the occurrence and the absence of the compounds in the leaves of *Balanites aegyptiaca*(table 7).

Characterization and Discussion of Isolated Compound

The compound was isolated and characterized from the methanolicextracts of the leaves of *Balanites aegyptiaca*. Structure elucidation of the compound was based on the spectroscopic data obtained for the compound [17-21].

Physical-chemical Characters:

Compound was obtained asyellowcolored pure oil withR_f valueof 0.43.BA-3is soluble in chloroform and ethyl acetate. The identity of the isolated compound was done chemically by Mg-HCl (Shinoda test).The formation of red color by Shinoda test indicated that the compound is flavonoid [22-24].

The IR Spectrum

The isolated compound showedthe absorption band at 3433cm^{-1} showed the presence of a hydroxyl group.Absorption band at 3119 and 3096cm^{-1} and medium absorption band at 2944cm^{-1} showed the aromatic C-H stretching. Absorption band at 1644cm^{-1} due tothe presence of carbonyl carbon anda strong absorption band at 1483cm^{-1} showed the presence of C=C ring stretching. The medium absorption band at 1088.5cm^{-1} showed the presence of-C-O-C- linkage bending. The medium absorption band at 1047 & 1033 cm^{-1}showed = C-H bending .

NMR Results

The ^{1}H NMR spectrum of isolated compound was showed three methyl signals of methoxygroups (three singlets) at δ3.863, 3.910 and 4.053 ppm, peaksat δ 6.606 (s, 1H), δ 7.022-7.044 (*d*, 1H) and 8.072 and 8.089(*d*, 1H) correspond to methine protons and 12.935 ppm indicated the presence of hydroxyl group. The ^{13}C NMR indicated that the compoundhas 18 carbons. The spectrum shows three methoxycarbons at δ55.44, 60.15 and 60.90 ppm. The three signals in the chemical shift at 93.13, 114.07 and 130.18 ppm, which belongs to methine carbon and one signal at δ 179.19 ppm due to carbonyl group.The remaining 12 carbons are quaternary carbons.

The DEPT spectrum revealed that its carbon skeleton was composed of three methyls, three methines and twelve quaternary carbons.

Moreover, the final structure of the above prediction was elucidated using 2D NMR (HMQC and HMBC) correlation spectra as follows. The HMQC was used to correlate protons with carbon atoms to which they are directlyattached. In addition to these the HMBC together with proton multiplicity gave so valuable information to identify and define the connectivity of different groups in the compound.

Hetronuclear **M**ultiple**Q**uantum **C**orrelation (**HMQC**) experiment correlates the chemical shift of proton/s with the chemical shift of directly bonded carbon. The HMQC spectrum of showed three protons at δ 3.863 (m) attached to C3'' of methoxyδ 60.15, three protons at δ 3.910 (m) connected with C2'' of methoxyδ 55.44,three protons at δ 4.053 connected with C6'' of methoxy δ60.90, a proton at δ 6.606 connected with C3'- δ 93.13, protons at δ 7.022-7.044 (d) attached to C-6 δ 114.07and a proton at δ 8.072-8.089(d) connected with C-7 at δ 130.18.

The methine proton of H-6 correlates with carbons C5 and C10; Protons of methine H-3'alsocorrelate with quaternary carbons C2', C4', C5' and C6'; Methine proton on H-7 correlates with C9and C10. On the basis of all the NMR data above structurewas attributed to isolated compoundwhich belongs to a group of natural product called flavonoids.

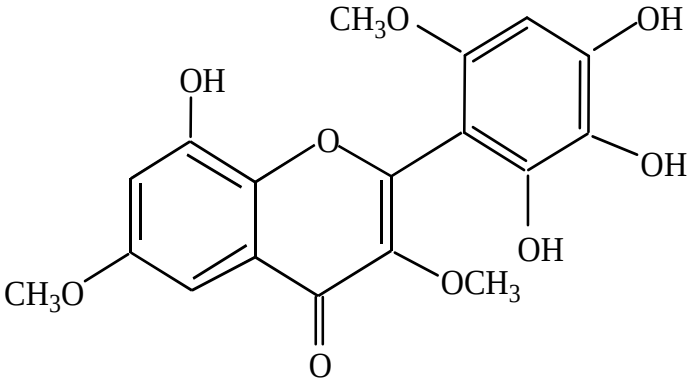

Figure 1. Proposed structure of isolated compound

CONCLUSIONS

From this study it can be concluded thatPhytochemical screening of the methanol extract of *Balanites aegyptiaca*revealed the presence of steroids, phenolic, terpenoids, tannins, saponin, flavonoids, alkaloids, amino acids and proteins and fixed oil and fats in the methanolic crude leaf extracts of this plant. Isolated compound was tentatively characterized as flavonoids using 1D and 2D NMR and IR spectra. Itscomplete structural elucidation requires the use of more efficient analytical techniques like high performance liquid chromatography (HPLC) and mass spectroscopy (MS).

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