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Iridoid Glycosides Isolated from *Combretum molle* Stem Bark Aqueous Methanol Extract

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Abstract: Plant-derived constituents serve as supplements to modern drugs. In practice, most of these drugs especially those derived from' bitters offer effective treatment. This is not surprising because about 40% of all pharmaceutical presently in use are derived from natural sources. The study investigated *Combretum molle* for its active constituents as well as determining the correlation between activity of the crude extract and/or the pure chemical constituents and the medicinal uses of the plant. Both column and thin layer chromatography were employed in the isolation of active principles from the aqueous methanol portion after the solvent partitioning. The active principle isolated thereof was identified using ultraviolet light and mass spectroscopy. Based on both the UV absorption and spectral data (λmax, nm) of 211.00 nm, 227nm and 229.00 nm, iridoid glycoside was the most likely isolated constituent in the column fractions of the extract.

Key words: Iridoid · Glycoside · *Combretum molle* · Stem · Bark · Thin Layer Chromatography

INTRODUCTION

Today, phytochemical and pharmacological investigations have added a great deal to the knowledge of the basis for the use of medicinal plants by revealing the presence of active principles as well as their actions on human and animal systems [1]. Investigation in the field of pharmacognosy and pharmacology have thus supplied valuable information on medicinal plant with regard to their availability, botanical properties, method of cultivation, collection, storage, commerce and therapeutic uses [1]. All these have contributed towards their acceptance in modern medicine and their inclusion in pharmacopoeias of some nations, thus it is necessary to increase the efforts in collecting and screening plants for the development of novel and environmentally safe medicinal agents [1].

Plant-derived remedies serve as supplements to modern drugs [2]. In practice, most of these drugs especially those derived from' bitters offer effective treatment. This is not surprising because about 40% of all pharmaceutical presently in use are derived from natural sources (plants, fungi and other microorganisms, animals, etc.), either used directly as such, or with some modifications [3, 4]. Unfortunately, the use of crude plant extracts without any scientific evaluation of their constituents, efficacy/possible side effects, could lead to serious complications [5]. Thus, there is a need to evaluate and establish the plant constituents and a scientific rationale for the handling and use of the traditional medicinal plants through validated scientific research.

Combretum molle have been found to possess good antibacterial, anthelmintic and antifungal effects, as well as having applications in traditional medicine [6]. Simon *et al*. [7] also reported the anthelmintic effect of the aqueous methanol extract of *C. molle* extract in rats.

Combretum molle R.Br Ex G.Don (also called Soft-leaved C*ombretum*, or Velvet bush willow*)* is a tree with large, straighter pole than most species of *Combretum,* distinguished by its rough bark and dense crown. It is locally referred to as wuyan damo (Hausa), damoruhi (Fulani) and aragba (Yoruba); occurs throughout tropical Africa and on the Arabian Peninsula in areas where woodlands and wooded grasslands predominate, often forming pure stands on hillsides [8].

This study therefore aims at carrying out chemical investigations of *C. molle* for its active constituents as well as determining the correlation between the purported activity of the crude extract and/or the pure chemical constituents and the medicinal uses of the plant.

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MATERIALS AND METHODS

The stem bark of *C. molle* was collected in Zaria, Nigeria. The plant was properly identified at the Herbarium Section of the Biological Sciences Department, Faculty of Sciences, Ahmadu Bello University, Zaria and a voucher number. 2797 was deposited. The plant material was air dried and ground to a powder (1 kg) using a mortar and pestle. The extract was prepared by Sohxlet extraction in absolute methanol (Sigma Aldrich 32213) with a 10:1 solvent to dry weight ratio [9-13] and the crude methanol extract (CME) was concentrated to dryness using a vacuum evaporator.

The method of Brain and Turner [14] was adopted for the partial purification (partitioning) of the dried CME. Briefly, 316 g of the dried CME was suspended in 500 ml of water.

The aqueous methanol extract was partitioned with three portions 250 ml each of petroleum ether (Sigma Aldrich 32213) using separating funnel. The petroleum ether portion was carefully separated into a clean 1000 ml beaker. Subsequently, the aqueous methanol portion thereof was partitioned with three (3) portions of 250 ml each of chloroform (Sigma Aldrich 32213); followed by three portions of 250 ml each of N-butanol (Sigma Aldrich 32213) leaving behind the aqueous methanol. The portions were subsequently referred to as petroleum ether, chloroform, N-butanol and aqueous methanol portions respectively. The separated portions of the extract were evaluated by thin layer chromatography (TLC) according to the methods described by Agrawal and Paridhavi, [15].

Fractionation, Isolation and Identification of Constituents from Aqueous Methanol Portion (AMP): A bioassay guided fractionation was carried out on the aqueous methanol portion in an attempt to isolate and identify its active component.

The wet packing technique described by Agrawal and Paridhavi [15] was employed. Fifty grams (50 g) of the silica gel G 60-200 microns (Interchin, France) was suspended in 250 ml of chloroform (the mobile phase) in a beaker and poured into the glass column (30 cm long with an internal diameter of 2 cm). The silica gel/chloroform suspension was continuously run through the column until the stationary phase (silica gel G) settled uniformly; care was taken to avoid the development of suspended silica gel-air bubbles and cracks in the column. One gram (1 g) of the solute (the aqueous methanol portion) was mixed with one gram (1 g) of sand (Courtin and Warner Ltd. Lewes, Sussex England), packed in a cotton wool and then pushed into the column until it was in contact with the stationary phase. Using the gradient elution technique [15], 30 ml each of the various eluting solvents; chloroform, chloroform-methanol mixtures with increasing amount of methanol; were ran through the packed column one after the other. About 25 ml of the eluents was collected for each solvent in a sample bottle. The fractions were monitored on TLC and fractions with the similar retention factor (R_f) values were combined.

Before performing TLC, the solvent system needed to separate the constituents was determined. Several solvent system combinations at different ratios (N-butanol; acetic acid; water (8:1:1), Chloroform; Methanol (4:1), Ethyl acetate; Methanol (1:4), Chloroform; Acetone (3:1), Ethyl acetate; pyridium; water (5:1:4) were tested on the crude methanol extracts; the solvents and ratio that best separated the crude methanol extract into different bands (layers) of separation was utilized as the solvent system for TLC. At the end of the trial, n-butanol; glacial acetic acid; water (8:1:1) system was found most suitable for separating the extract into different constituents layers on TLC. Thus, this was chosen as the experimental solvent system for the purpose of this study.

TLC Monitoring of Column Fractions: The nine fractions were obtained from the column fractionation (fractions 1-9) were monitored on TLC in n-butanol, glacial acetic and water (8:1:1), similar fractions were pooled together based on their retention factor (R_f) result. At the end, two major fractions were obtained; Fraction A (comprising of fractions 1-4) which did not respond to the mobile phase and fraction B (comprising of fractions 5-9) which responded to the mobile phase and gave similar retention factor (Rf) values.

$$Rf = \frac{\text{Distance from origin to the point of maximum intensity}}{\text{Distance from origin to the solvent front}}$$

Isolation of Constituents on Thin Layer Chromatography (TLC): Aluminium sheets and glass-backed TLC plates (20 x 20 cm; Merck, silica gel 60 F254) were used for the isolation of compounds. The plates were divided into sizes of 10 cm x 1.5 cm. A light pencil line was drawn 1 cm from the bottom and top edge of the chromatographic plate. 6 mg of column fraction B was dissolve in 60 ml of absolute methanol and placed as preparatory on 40 TLC plates (10 cm x 1.5 cm.) using a 10 µl capillary which delivers approximately 10 µg/spot onto the plate until

Plate I: Spotted TLC plate (A) with extract and developed in locating agent, B= bands of constituent located, C= spot base line

each plate contains 150 µg and subsequently placed in the eluting solvent (30 ml of n-butanol, glacial acetic acid, water; ratio of 8:1:1) in a TLC tank which was filled to a depth of 0.5 cm. The solvent migrated upward onto the TLC plate until the pencil line drawn across the top edge (solvent front) was reached. The plates were then removed from the chamber and air-dried. A portion of the plate (1 cm) was cut off using a glass cutter and sprayed with a detecting reagent (anisaldehyde, methanol, concentrated sulphuric acid; ratio of 90:10: 10) in order to visualized the constituents on the eluted plate after heating for 3 min at 110°C in an oven (Fischer Scientific, China) (plate I). The plates were also visualized under camac universal TL-600 ultraviolet (UV) light at 360 nm and 254 nm and the flourescin (360 nm) or quenching (254 nm). Compounds were marked and the spot (layers) were outlined with a pencil.

Concentration of Compounds Isolated on TLC: The detected compounds under the UV spectra (Camac universal TL-600 spectrophotometer) were separately and carefully scrapped off from the plates into different 250 ml beakers based on bands (colour) of separation. The silica/constituent mixture were dissolved in 100 ml of 70% methanol, shaken and poured into 10 ml centrifuge tubes and centrifuged at 5000 rpm (SG 1.2) for 5 min (Hermle. GmbH & Co. FR. Germany). The supernatant which contains the active constituent was decanted into a 150 ml beaker and allowed to air-dry at room temperature.

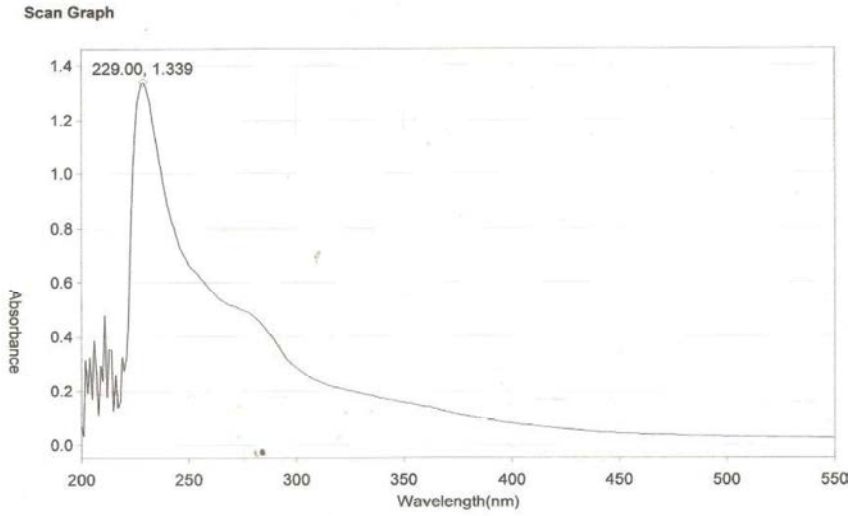
Identification of Isolated Constituent Using Mass Spectroscopy: Mass spectroscopy analysis was carried out on a UV Visible Double Beam Spectrophotometer (Hellions Zeta. Thermo Scientific U.K- Plate VIII) fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 µm). The temperature in the oven was set at 3°C/min for maximum of 60°C. Helium gas was used as a carrier and delivered at a flow rate of 2 ml/min. The spectrophotometer was coupled to a Hewlett-Packard 6890 mass selective detector with MS operating parameters specifications of 70 eV ionization voltage; 200°C ion source temperature; lambda max and wave length. 0.1 mg of the isolated constituent was dissolved in 5 ml of absolute methanol; 2 ml of this mixture was placed in the capillary column, inserted into the hp-chromatograph and ran to obtain the lambda max and the wave length.

RESULTS

Column Fractionation of Aqueous Methanol Portion: Concentration of fraction A (fractions 1 to 4) to dryness gave a yield of 120 mg (12%), while concentration of fraction B (5 to 9) yielded 800 mg (80%). The five fractions (5 to 9) that were pooled together to give fraction B had similar retention factors (RF) (Table 1).

Isolation and Concentration of Compound on TLC: Three spots (A, B, C) were located on TLC after spotting 6 mg of column fraction B (Plate II). After scrapping,

Plate II: TLC showing the location of the active constituents A, B and C scrapped



Results Table - scan004,C,Cycle01

nm	A	Peak Pick Method
229.00	1.339	Find 8 Peaks Above -3.0000 A
		Start Wavelength 200.00 nm
		Stop Wavelength 550.00 nm
		Sort By Wavelength
Sensitivity	Auto	

Page 1, Scan Graph

Fig. 1: Scan graph of spectrum of isolated constituent A

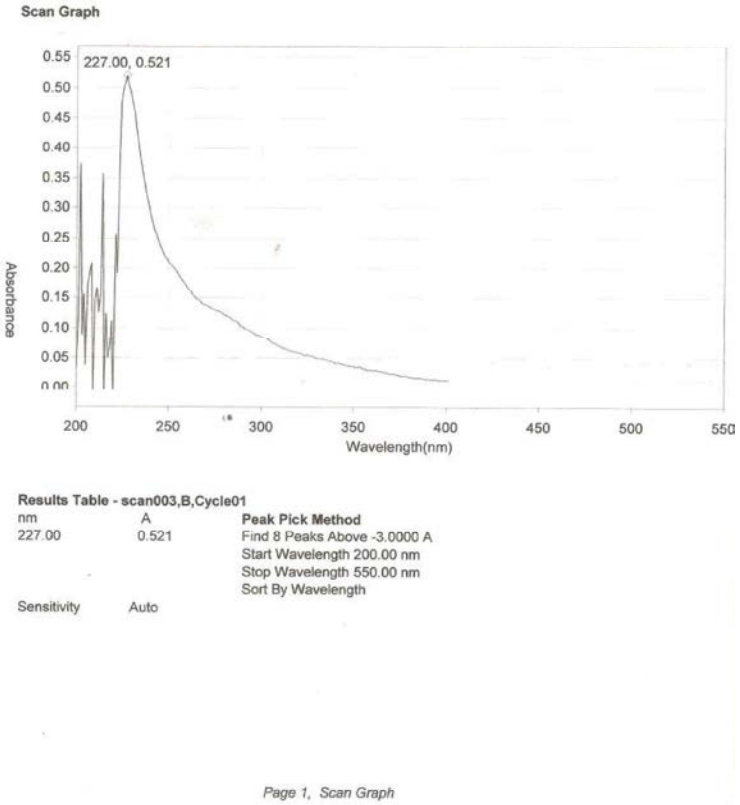


Fig. 2: Scan graph of spectrum of isolated constituent B

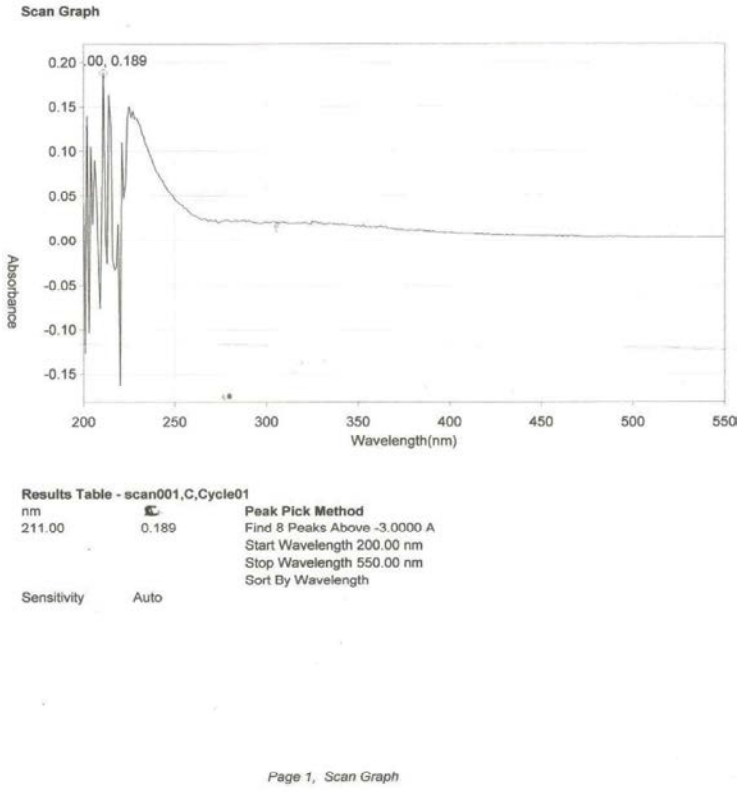


Fig. 3: Scan graph of spectrum of isolated constituent C

Table 1: Quantity and percentage yield of column fractionation of aqueous methanol portion.

Column fraction	Quantity after Conc (mg)	% yield	Response to solvent system	Rf value
1=Pure chl	5	0.5	-	-
2=Chl/Me (9:1)	22	2.2	-	-
3=Chl/Me (4:1)	35	3.5	-	-
4=Chl/Me (7:3)	58	5.8	-	-
5=Chl/Me (3:2)	80	8.0	+	0.470
6=Chl/Me (5:4)	122	12.2	+	0.465
7=Chl/Me (1:1)	186	18.6	+	0.471
8=Pure Me	220	22.0	+	0.468
9=20% H_2O in Me	192	19.2	+	0.464

Key;- Chl= chloroform; Me= methanol; H_2O= water;%= percentage; Qtty= quantity; Conc= concentration; Rf=rentention factor

Note; fractions 1-4 are pooled together to give column fraction A (CFA) while fractions 5-9 were pooled to give column fraction B (CFB)

Table 2: Output/percentage yield of constituents isolated on TLC after concentration

Constituents	Quantity (mg)	Percentage yield
A	1.2	20
B	0.6	10
C	0.8	13.3

dissolving in 100 ml of 70% methanol, centrifuged and concentrating the supernatants to dryness, spot A, B and spot C gave 1.2 mg, 0.6 mg and 0.8 mg yields of active constituents respectively (Table 1).

Identification of Isolated Constituents' Mass Spectrum: Constituents A, B and C were obtained as amorphous powders. The mass absorption spectrum exhibited maxima (λmax, nm) in methanol of 229 nm (A), 227 nm (B) and 211 nm (C) (Figs 1, 2 and 3).

DISCUSSION

Based on the Mass absorption spectral data (λmax, nm) of 211-C, 227-B and 229.00-A nm, iridoid glycosides were the most likely isolated constituents in the column fraction of the aqueous methanol portion of *C. molle* stem bark methanol extract. In similar studies, iridoid glycosides with the same spectral data were isolated from *Globularia dumulosa* and *Globularia davisiana* by Hasan *et al*. [16] and Calis *et al*. [17] respectively. An iridoid glycoside isolated from *Veronica pectinata var. glandulosa* and *Nepeta septenarenata Eremb* showed spectral data of lambda max of 210 nm, 299 nm and 331 nm [18] and lambda max of 200 nm (aucubin), 220 nm (ajugol) and 224 nm (ajugoside) [19] which is consistent to that isolated from the plant under study.

Iridoids glycosides (a cardiac glycosides) represent a large group of cyclopen-tano[*c*]pyran monoterpenoids this includes; urphoside, aucubin, catalpol, benzoyl, veronicoside, p-hydroxybenzoyl, catalposide, protocatechuoyl, verproside, vanilloyl, amphicoside, veratroyl and 6-*o*-veratroyl catalpol [18]. These iridoids are found as natural constituents in a large number of plant families. Iridoid glucosides have biogenetic and chemotaxonomic importance since they provide a structural link between terpenes and alkaloids [20]. A bicyclic H-5/H-9 β β-*cis*-fused cyclopentanopyran ring system is the most common structural feature of these substances; however several enantiomeric iridoids also exist in nature suggesting their complex stereochemistry [21, 22].

CONCLUSION

The investigation of chemical compounds from natural products is fundamentally important for the development of new drugs, especially in view of the vast worldwide flora. Based on the results presented in this work *C. molle* is a good candidate for the development of effective pharmaceutical compounds which will serve as a better alternative to chemical based pharmaceuticals.

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