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Full Length Research Paper

Isolation and characterization of anthraquinone derivatives from *Ceratotheca triloba* (Bernh.) Hook.f.

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Following a complete chemical profile of methanolic extracts of *Ceratotheca triloba* roots, stems, flowers and leaves, the predominant compounds were isolated, and characterized by preparative thin layer chromatography (TLC) and identified by UV, ^{1}H NMR, ^{13}C NMR and EI-LC-MS.Three anthraquinone derivatives; 9, 10 anthracenedione; 1-hydroxy-4-methylanthraquinone;and 5,8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4, 9-dione and also a steroid; androst-5-ene-3, 17, 19-triol were isolated from the roots of *C. triloba*. Of these, 9, 10 anthracenedione and 1 hydroxy -4-methylanthraquinone showed antibacterial and anti-oxidant activity, and showed potent inhibition of the human topoisomerase II enzyme. This enzyme, in a normal cells causes the unwinding of the DNA and is also structurally similar to commercial anti-cancer compound mitoxanthrone which is also an anthracenedione.

Key words: Anthraquinone, 9, 10 anthracenedione, 1 hydroxy -4 methylanthraquinone.

INTRODUCTION

Anthraquinones are a class of natural compounds that consists of several hundreds of compounds that differ in the nature and positions of substituent groups (Schripsema et al., 1999). This class of compounds contains derivatives that consist of the basic structure of 9, 10 anthraquinone (Bajaj, 1999). Anthraquinones can be divided into alizarin and emodin types based on two main biosynthetic pathways. The alizarin types are formed through chorismate/ō-succinylbenzoic acid pathway and only have one of the rings unsubstituted (Koblitz, 1988; Van der Berg and Labadie, 1989). These anthraquinones are found in the family of plants known as Rubiaceae (*Rubia, Morinda, Galium* and *Cinchona*) (Korunaglo et al., 1992). The emodin types are formed through the polyketide pathway (acetate-malonate pathway) and have both rings substituted. Anthraquinones are widely applied in medicine, food and the dye industry. In the pharmaceutical industry, the natural and synthetic derivatives of 9, 10 anthraquinone are beneficial to mammals and humans as they can display antibacterial, antitrypanosomal and antineoplastic activities (Heyman et al., 2009; Tarus et al., 2002; Velez-Cruz and Osheroff, 2004). In this study the initial approach of data mining did not give any information related to the chemical constituents or the biological actions of *Ceratotheca triloba*, therefore we took the chemical approach to identify the predominant compound/s. In our current efforts of searching for new bioactive agents from indigenous plants, the chemical constituents of the roots of *C. triloba* were investigated which led to the isolation of three new anthraquinones (1-3) and steroid androgen, androst-5-ene-3, 17, 19-triol (4). Herein, we report the isolation and structure elucidation of the new compounds by extensive spectroscopic techniques including UV-Vis, IR, HR-EI-MS and NMR (COSY, HMQC, HMBC and DEPT).

MATERIALS AND METHODS

Preparation of plant material

C. triloba (Bernh.) E. Mey. ex. Hook.f. was collected in Durban,

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Kwazulu Natal, South Africa, and identified by using available floral keys. A voucher specimen (Baijnath sn.) was deposited in the Ward Herbarium, University of Kwazulu-Natal (Westville Campus). The plant portals were carefully examined and old, insect-damaged, fungus-infected roots were removed. Healthy roots, stems, leaves, flowers and seed pods were spread out and dried in the laboratory at room temperature for 5 to 8 days or until they broke easily by hand. Once completely dry, plant material was ground to a fine powder using a Wareing blender. Larger quantities were crushed to a fine powder of 1.0 mm diameter using a Retsch Mühle mill at the Department of Biotechnology and Food Technology (Durban University of Technology). Material was stored in a closed container at room temperature until required.

Extraction

The extraction protocol of Harborne and Harborne (1998) was modified and used in this research. This method separates the plant metabolites into different groups based on their polarity. The fresh plant material was homogenized for 5 min in methanol/water (4:1) and then filtered. This resulted in the filtrate and residue. The residue was discarded and the filtrate was concentrated to 1/10 volume and acidified with 2 M H_2SO_4. The acidified filtrate was extracted with chloroform (3X) and this resulted in the chloroform partition and the aqueous acid layer. The chloroform partition would contain all the moderately polar compounds (terpenoids and phenolics). The aqueous acid layer was then basified to pH 10 with NH_4OH and further extracted with chloroform: methanol (3:1) to yield the polar and basic extract (mostly alkaloids). Extraction was initially performed using a method prescribed by Harborne and Harborne (1998). The principle of separation of bioactive molecules was based on the dissolution of these molecules in solvents of differing polarities.

Phytochemical screening of plant extracts

Phytochemical screens for tannins, phlobatannins, saponins, flavanoids, steroids and terpenoids were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

Separation of compounds in the different plant portals by TLC

Thin layer chromatography was on Merck thin layer chromatography (TLC) F_{254} or silica gel 60 plates using hexane: ethyl acetate (9:1) as eluent. Streaking of polar components was minimized by the addition of 1% ammonium chloride to the mobile phase solution. Samples (5 to 10 µl of a 100 mg extract/ml solution) were spotted and run without delay to minimize the possibility of oxidative or photo-oxidative change. Separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camag Universal UV lamp TL-600). Plates were also sprayed with ρ-anisaldehyde (5% anisaldehyde in 5% sulphuric acid in ethanol) and heated for 2 to 5 min at 100°C to allow for development of colour changes (Carr and Rogers, 1986).

Purification of crude extracts

Column chromatography

Silica gel (Kieselgel 60, 0.015-0.04 mm) was suspended in required solvent and left for approximately 2 h to swell after which it was poured into the column. The fraction obtained during solvent/solvent extraction was suspended in the minimum amount of the particular solvent (30 g silica/1g sample) in which it would dissolve and filtered to remove impurities and any large particles which could cause diffusion problems whilst developing the column. This fraction was applied to the top of the column using a pipette with great care as not to disturb the top of the column. After application, the solvent flask was raised to facilitate solvent flow into the column and was run using gravitational force. A concentration gradient of eluents was used. With the N-hexane fraction, a 500 ml mixture of hexane: ethyl acetate (1:1) was used, gradually introducing a mixture of hexane: ethyl acetate (2:1). Finally 100% hexane was added to elute any components that could not be moved with the other solvents. The column was left to run overnight at a flow rate of 0.5 ml/min.

Analysis and concentration of fractions

As soon as column chromatography was completed, test-tubes were placed under a stream of nitrogen to facilitate concentration of the fractions for TLC analysis and bioassay. After approximately 40% of the volume of the eluent had evaporated, every second fraction was analyzed by TLC using 0.1% of each fraction. In some cases test-tubes were not pre-weighed and the fractions were not dried, therefore it was not possible to determine the exact concentration applied to the TLC plates. Fractions were analyzed using Hexane: ethyl acetate (9:1). Ethyl acetate/Methanol/Water (45:5:4.4) was also attempted but with poor results. Separated components were sprayed with ρ-anisaldehyde reagent.

From TLC results, fractions were combined according to their separation profile. Combined fractions were placed under an air current to facilitate drying. Once dry, the components were weighed to calculate the total mass extracted, dissolved in the minimum amount of acetone possible and transferred to a glass vial with a screw top to prevent evaporation.

Preparative TLC

Fractions were dissolved in 100 µl of solvent in which it would dissolve and applied in a band across the preparative TLC plate (Silica gel 60 F_{254}) starting and ending at least 1 cm from either side. The plate was developed, repetitively (at least three times) in the mobile phase and the bands visualized under ultraviolet light (254 and 360 nm). A small section on the side of the plate was sprayed with vanillin and heated with a heating gun, whilst protecting the rest of the plate with foil. Components were easily visualized and marked using a soft 2B pencil. After spraying the plate with water to facilitate easier removal of components, the bands were scraped off the glass plate. The components were collected into separate beakers and crushed to a fine powder using a glass rod. The adsorbent powder was eluted with 5 ml acetone, depending on the quantity and pigment recovered by filtration using a sintered glass funnel. The process was repeated at least twice or until the powder regained its original white colour. This was followed by 1% acetic acid in methanol for a final rinse to remove any polar components not removed with acetone. Each component was collected into a separate vial and concentrated under a stream of cold air.

Melting point determination

Melting points were determined on Kofler hot-stage apparatus and are uncorrected.

UV/Vis and EI-LC-MS

The analysis was done on a waters thermabeam (TMD) system

Table 1. Gradient conditions on the Waters 2695 solvent delivery system.

Time	Flow	%C	%D	Curve
0.0	0.20	70	30	6
1.0	0.20	70	30	6
40.0	0.20	0	100	6
48.0	0.20	0	100	4
50.0	0.20	70	30	3
60.0	0.20	70	30	6

comprising of a 2695 Solvent Delivery System, a 2996 photodiode array (PDA) detector, column heater and TMD electron ionization mass spectrometry detector. Chromatographic separation was done on a waters Xbridge C_{18} column (150 x 2.1 mm, 3.5 µm) maintained at 40°C. The starting eluent consisted of water (containing 10 mM Formic acid) and Acetonitrile (70:30) at 0.2 ml/min. The gradient table of the chromatographic method is summarized in Table 1.
The photodiode array (PDA) detector was placed first in line and full scan spectra were collected between 200 and 600 nm at a sample rate of 1 spectrum per second and a resolution of 1.2 nm. The TMD detector was placed after the PDA detector and operated in positive scan mode (50 to 650 amu) with a gain of 10 collecting 1 spectrum per second. The nebuliser temperature was set at 90°C, the expansion region temperature at 80°C and the source temperature at 225°C. The total volume of post-column eluent was sent to the TMD detector and helium was used as nebulisation gas at 30 L/h. The TMD detector was tuned every day prior to starting an analysis run and caffeine was injected as test compound to ensure functionality of the total system. Injection volumes ranged between 1 and 10 µl depending on the concentration of the sample. For samples CTREM01 to CTREM03 the injection volume was 2 µl while the injection volume for CTREh was 10 µl.

IR, ^{1}H NMR and C^{13} NMR spectroscopy (COSY, DEPT, HSBC and HMQC)

Infrared spectroscopy was carried out on a Varian 800 FTIR Scimitar series utilizing a PIKE MIracle™ cell with KBr loaded lenses. Samples were run in ATR (attenuated total reflectance) mode. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR [Merck]. In the studies, $CDCl_3$ was used as the solvent of choice, although other solvents were also attempted, because of its good ability to dissolve a wide range of compounds. The samples were then pipetted into NMR tubes with the aid of a pasteur pipette and sent to Mr Dilip Jugjivan of the Chemistry Department, University of Kwazulu-Natal (Westville). ^{1}H NMR was run at 400 MHz and ^{13}Cat 75 MHz using the solvent signal tetramethylsilane (TMS), $(CH_3)_4Si$ as reference on a BRUKER Avance III NMR system. The spectra were interpreted by the aid of the COSY, DEPT, HSBC, and HMQC techniques.

RESULTS

Profile of compounds in plant portals

Using optimized separation and detection parameters, the components in the different portals of the plant were compared. The compounds varied from six in the stems and seed pods to 13 in the roots (Figure 2). A profiling of the Rf values from roots, flowers, leaves, seed pods and stems showed three common compounds at Rf values of 0.21, 0.34 and 0.38 represented as 1, 2 and 3 in Figure 1.

Major phytochemical classes in *C. triloba*

Phytochemical screening from the crude extracts showed the presence of phlobatannins, saponins, steroids and terpenoids. No flavonoids, tannins and cardiac glycosides were detected. These results are shown in Table 2.

Column chromatography

The root extract was passed through a silica gel column using hexane-ethyl acetate (90:10) as the mobile phase. Twenty fractions were collected and analyzed by TLC. The colour and Rf values were recorded. This enabled us to combine fractions with similar TLC profiles. Fraction number E6-E20 were combined and this resulted in 3 compounds (A-C), which were then further analyzed by TLC (Figures 2 and 3, respectively).

Preparative TLC of fractions A, B and C

Fractions A, B and C were dissolved in as little hexane as possible and applied to a preparative TLC plate. The three bands (I-III) were marked off using UV and visible light as a visual aid and then scraped off and extracted first with acetone, chloroform and then 1% acetic acid in methanol to remove polar components. Products were dried under a stream of cold air to avoid heat decomposition and stored in the fridge. Only the acetone fractions were subjected to the qualitative analysis chromatography-mass spectrometry (LC-MS) as the quantities extracted with chloroform and 1% acetic

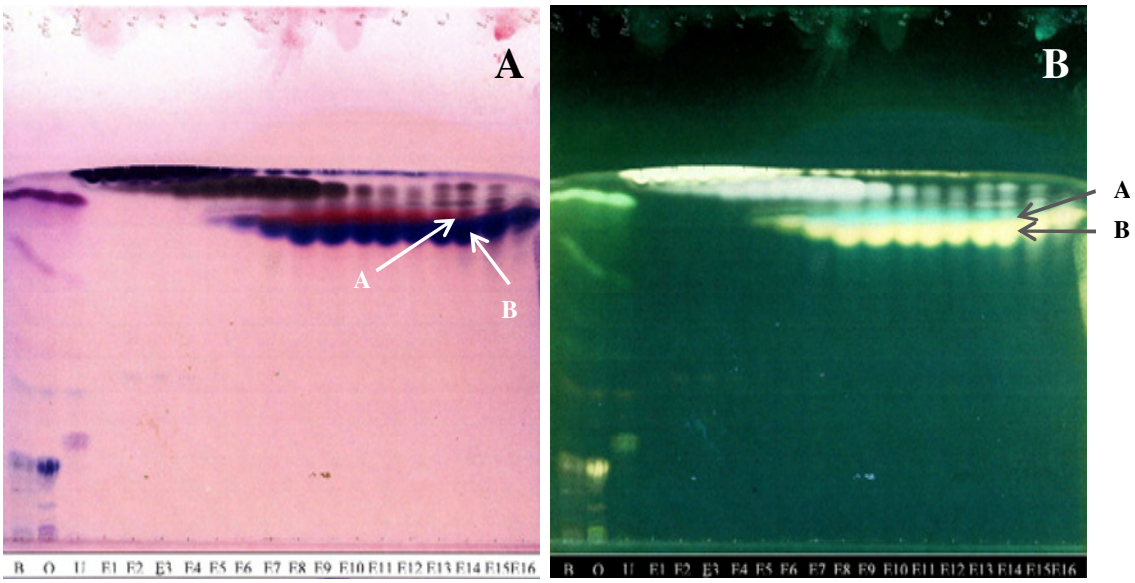

Figure 2. (A) TLC of the fractions (E1-E16), (B) Inverted or Negative image TLC of the fractions (E1-E16). The fractions were eluted with Hexane: ethyl acetate (90:10) and developed with ρ-anisaldehyde.

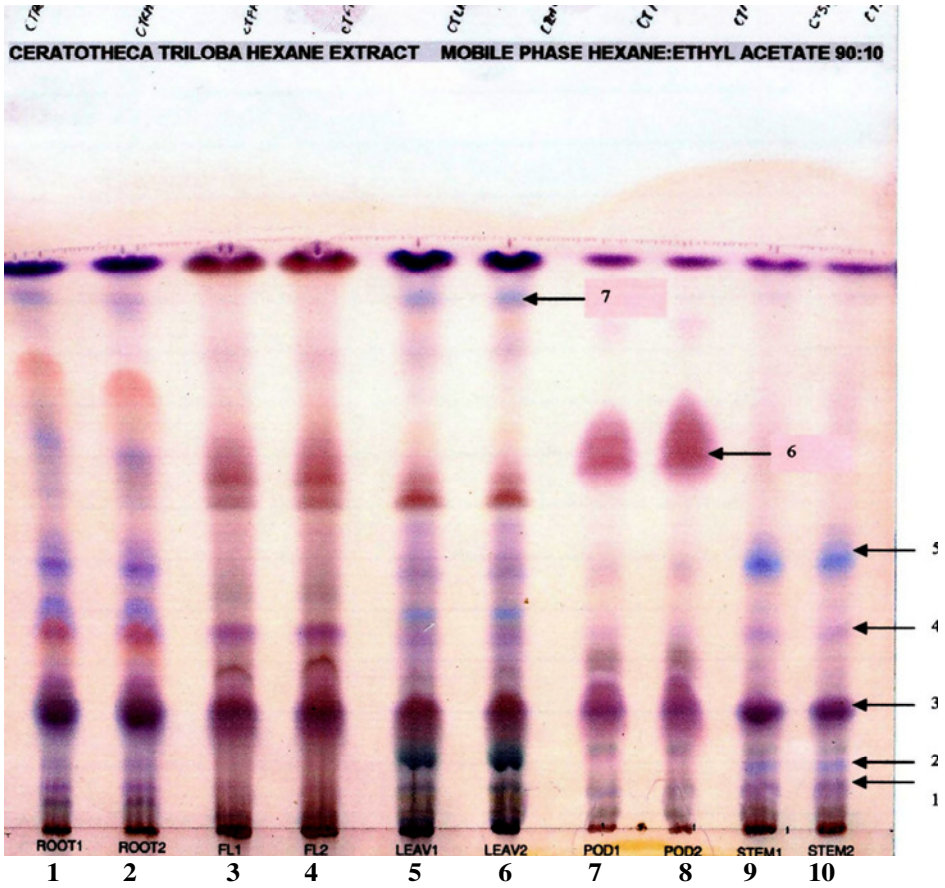

Figure 1. Separation of components from the different portals of *C. triloba*. Lane 1, 2 – root extract; Lane 3, 4 –flower extract; Lane 5, 6 –leaf extract; Lane 7, 8- seed pod extract and Lanes 9 and 10- stem extracts

Table 2. Major phytochemical compounds from crude extracts of *C. triloba*

Phytochemical constituents	Aqueous root extraction preparation 1	Aqueous root extraction preparation 2
Tannins	-	-
Phlobatannins	+ red precipitate at base of test tube	+ red precipitate at base of test tube
Saponins	+ formation of heavy emulsion	+ formation of heavy emulsion
Flavonoids	- no yellow colour change	- no yellow colour change
Steroids	+ Formation of a blue-green interface between layers	+ Formation of a blue-green interface between layers
Terpenoids (Salkowski test)	+ formation of a red-brown interface between layers	+ formation of a red-brown interface between layers
Cardiac glycosides (Keller-Killani test)	-	-

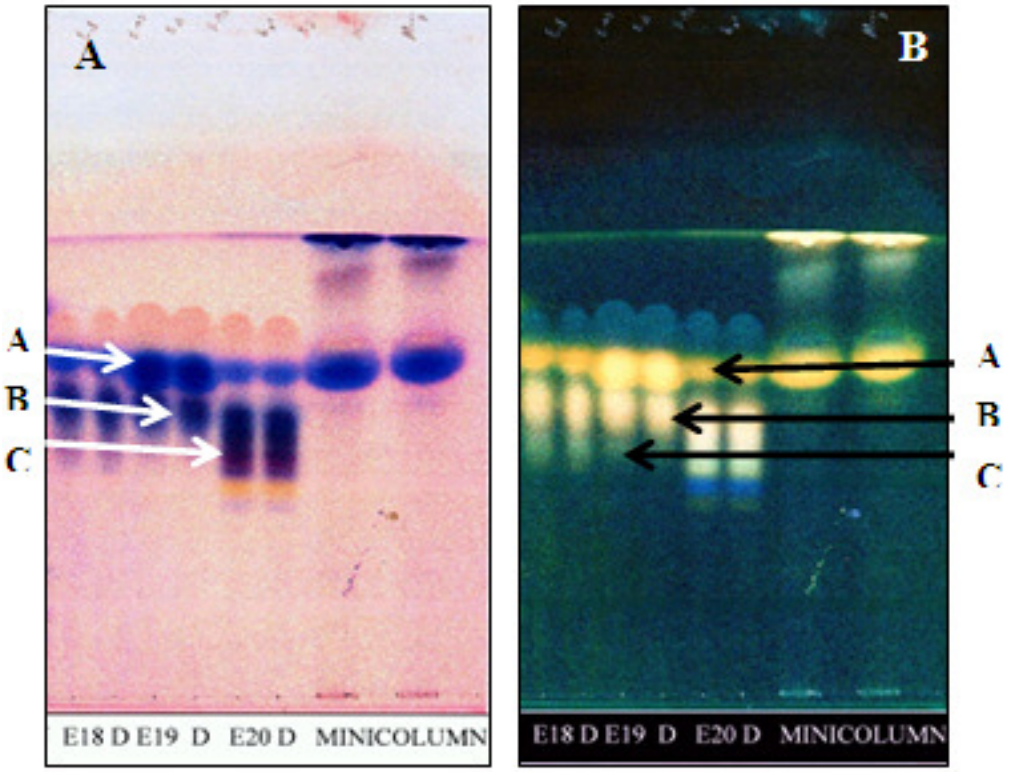

Figure 3. (A) TLC and inverted image (B) TLC of the fractions (E18-E20). The fractions were eluted with Hexane: ethyl acetate (90:10) and sprayed with ρ-anisaldehyde visualization spray reagent.

acid/methanol were insignificant.

Preparative high performance liquid chromatography and uv-vis spectroscopy of compounds isolated from *C. triloba*

Partially purified extracts were coded as CTREh01, CTREh02 and CTREh03 and further purified using preparative high performance liquid chromatography (HPLC). Figures 5, 6 and 7 illustrate the separation of pure compounds from CTREh01, CTREh02 and CTREh03 respectively. The compounds that were isolated from PTLC were not pure enough to determine their structures using LC-MS. All three extracts contained similar compounds at retention times of 22.133 and 4.500 min. Following preparative HPLC the compounds were separated by LC-MS as in Figures 4, 5 and 6.

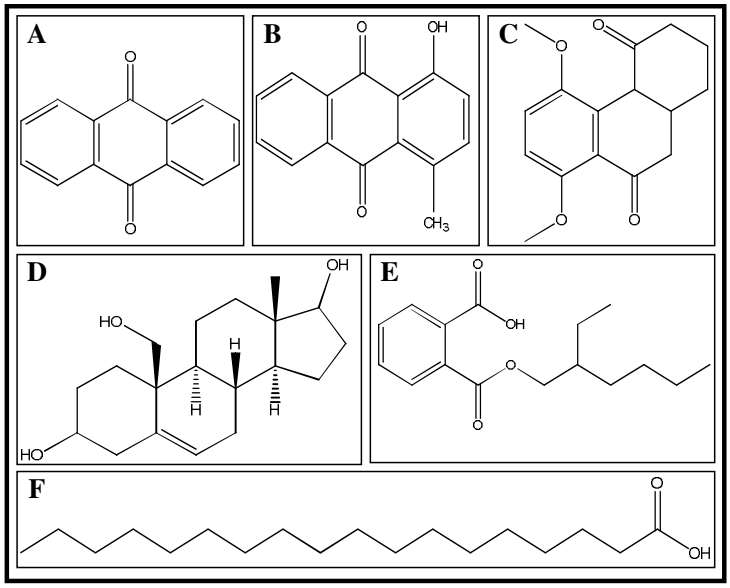

Figure 4. Six compounds isolated from *C. triloba* roots. Structures were confirmed with EI-LCMS. A- 9, 10 anthracenedione, B- 1-hydroxy-4-methylanthraquinone, C- 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione, D- androst-5-ene-3, 17, 19-triol, E- 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester and F- Octadecanoic acid.

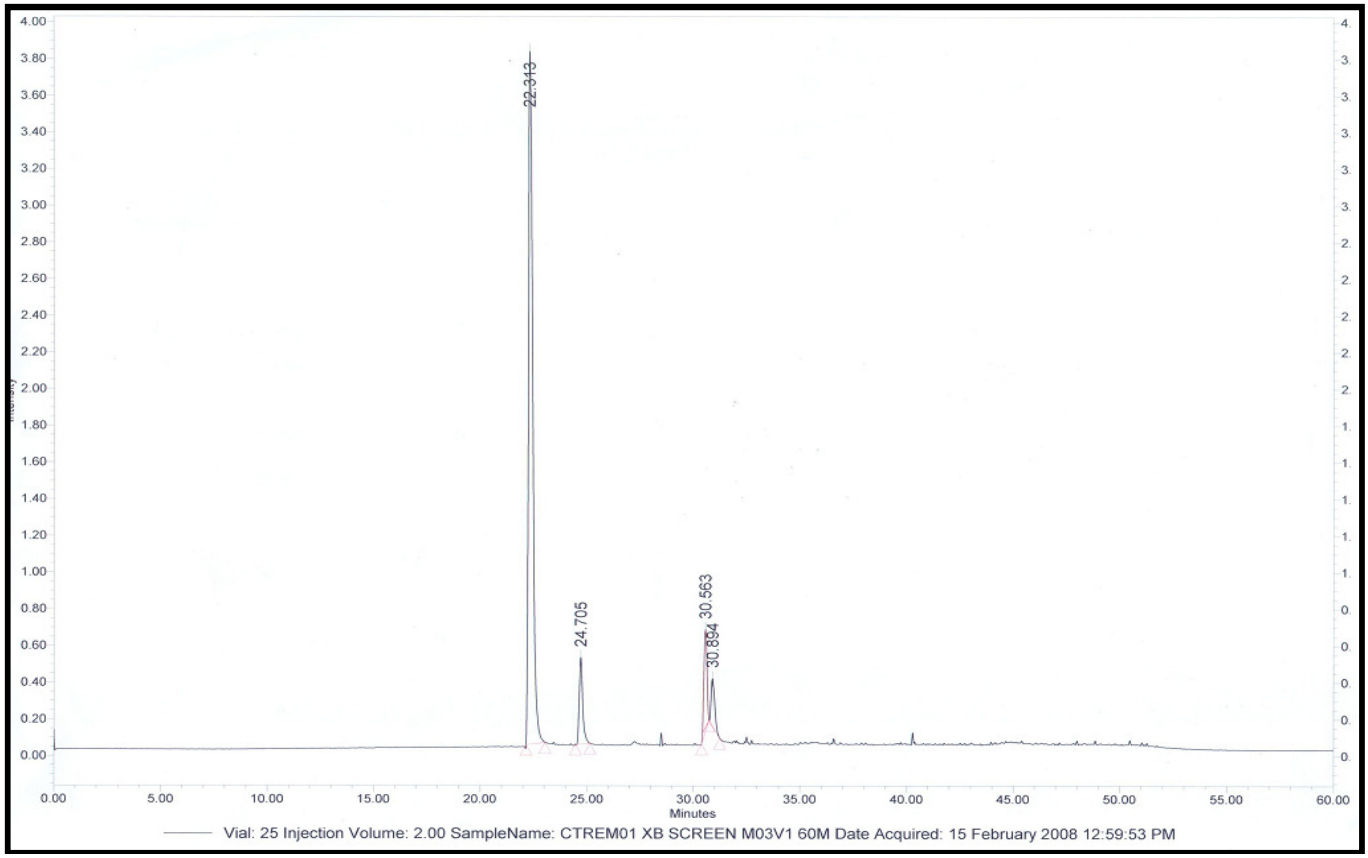

Figure 5. Preparative High Performance Liquid Chromatography (P-HPLC) of CTREh01 showing the presence of four distinct compounds at varying retention times. A-Retention time = 22.136, B- Rt = 24.705, C- Rt = 30.563 and F- Rt=30.894.

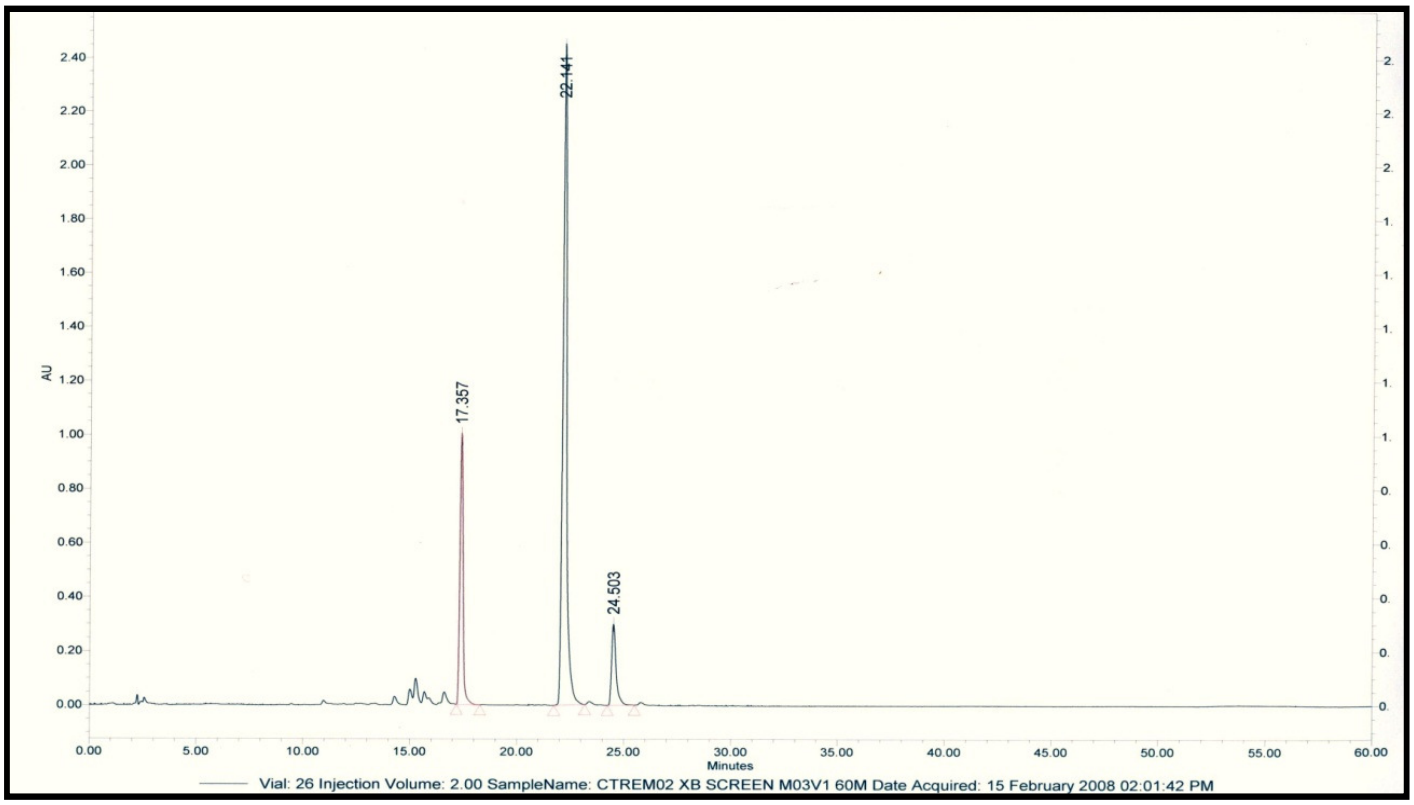

Figure 6. Preparative High Performance Liquid Chromatography of CTREh02 showing the presence of three distinct compounds at varying retention times. D-Retention time = 17.357, A- Rt = 22.141 and B- Rt = 24.503.

Liquid LC-MS analysis of purified components of *C. triloba*

LC-MS indicated six different compounds with varying structural differences representing anthraquinones and a steroid. Prevalent compounds belonged to the anthraquinones which included 9, 10 anthracenedione (Figure 7A), 1-hydroxy-4-methylanthraquinone (Figure 7B) and 5, 8- dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione (Figure 7C). The steroid isolated was identified as androst-5-ene-3, 17, 19-triol (Figure 7D). Two other compounds were also isolated 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester and octadecanoic acid.

Identification and verification of compound CTREh01-B

Final confirmation of the isolated compound as 9, 10 anthracenedione was achieved using EI-LCMS as well as a range of NMR experiments. The H^1 NMR, HSQC, HSBC and DEPT spectra of 9, 10 anthracenedione was consistent with the molecular formula $C_{14}H_8O_2$. The IR spectra showed absorption bands for the conjugated carbonyls (u 1590 cm^{-1}) and the aromatic rings (u 1590 and 1480 cm^{-1}). H^1 and ^{13}C NMR results are listed in Table 3. The H^1 NMR spectrum revealed four sets of overlapping multiplets integrating two protons at 7.7833-7.7988 and 8.275-8.3102, indicating a typical aromatic ring (Figure 7A). The H^1 NMR spectrum also revealed the presence of one set of a meta-coupled doublets at δH 7.79 (d, J=3.30 Hz, H-4) and at δH 8.30 (d, J=3.36 Hz, H-3).The HMQC, HMBC and DEPT analysis revealed the 9, 10 anthracenedione as a symmetrical structure with the absence of chelated hydroxyl and methyl groups. ^{13}C NMR revealed 14 carbon signals which were sorted by HSQC techniques into 12 quartenary carbons and two carbonyl groups detected at δ_C 183.17.

9, 10 anthracenedione (CTREH-01B): Obtained as yellow needles (hexane); mp: 213.50 -214.10°C; Chemical formula: $C_{14}H_8O_2$; Molecular weight: 208.2 g/mol; UV ($CHCl_3$) λ_{max} (log ε – molar absorption coefficient): 205.2 (1.63), 256.9 (1.72), 329.2 (1.83) HPLC- elution time (H_2O: Acetonitrile) 70:30 – 24.503-24.505; Mass spectral data: *m/z* – 62.90, 165.00, 193.10, 221.20, 222.10, 223.20, 247.10, 248.10, 273.00, 290.10 and 288.10.

Identification and verification of compound CTREh01-A

The H^1 NMR, HSQC, HSBC and DEPT spectra of

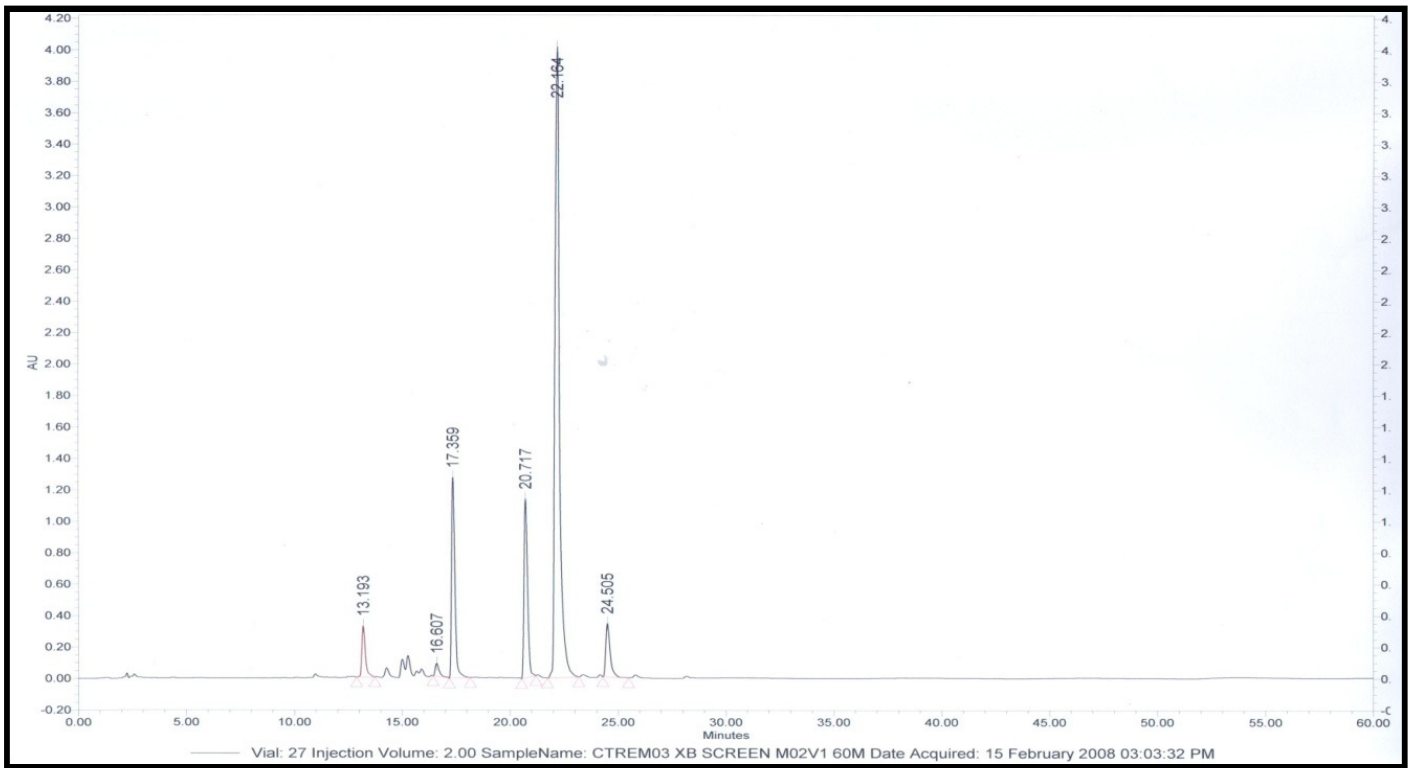

Figure 7. Preparative High Performance Liquid Chromatography of CTREh03 showing the presence of four distinct compounds at varying retention times. D-Retention time = 17.359, E- Rt = 20.717, A- Rt = 22.164 and B- Rt = 24.505.

Table 3. ^{13}C and H^1 NMR spectral data for 9, 10 anthracenedione.

Atom Index	^{1}H NMR shifts/ δ_H	^{13}C NMR shifts/ δ_C
1	7.7978 (1, 1H, *m*)	127.24
2	8.3102 (2, 1H, *m*,)	133.52
3	8.3109 (3, 1H, *m*)	133.52
4	7.7895 (4, 1H,*m*),	127.24
5	-	134.13
6	-	183.17
7	-	134.13
8	7.7833 (8, 1H,*m)*	127.24
9	8.2958 (10, 1H,*m)*	133.52
10	8.2875 (10, 1H,*m)*	133.52
11	7.7750 (11, 1H,*m)*	127.24
12	-	134.13
13	-	183.17
14	-	134.13

m- multiplets

1-hydroxy-4-methyl anthraquinone was consistent with the molecular formula $C_{15}H_{10}O_3$. The IR spectra showed absorption bands for a hydroxyl group (u 3450 cm^{-1}), the conjugated carbonyls (u 1590 cm^{-1}) and the aromatic rings (u 1590 and 1480 cm^{-1}). H^1 and ^{13}C NMR results are listed in Table 4. The hydroxyl proton at δ_H 5.35 exhibited heteronuclear interactions with the carbon atoms at δ_C 145.29 (C-1, $^2J_{CH}$), 127.49 (C-14, $^3J_{CH}$) and 127.14 (C-2, δ_H 7.24, $^2J_{CH}$).

These data indicated the location of the hydroxyl and

Table 4. ^{13}C and H^1 NMR spectral data for 1-hydroxy-4-methyl anthraquinone.

Atom index	^1H NMR shifts/ δ_H	^{13}C NMR shifts/ δ_C
1	-	145.29
1a	5.025	-
2	7.24	127.14
3	7.55	134.94
4	-	133.92
4a	2.5086	21.91
5	-	133.60
6	-	183.00 (carbonyl groups)
7	-	133.39
8	7.77	127.44
9	8.27	133.39
10	8.26	134.04
11	7.76	127.16
12	-	131.28
13	-	183.44 (carbonyl groups)
14	-	127.49

methyl groups at carbon atoms C-1 and C-4 respectively. The HMQC, HMBC and DEPT analysis revealed the 1-hydroxy-4-methyl anthraquinone as a non-symmetrical structure with the presence of chelated hydroxyl and methyl groups. ^{13}C NMR revealed 16 carbon signals which were sorted by HSQC techniques into 15 quaternary deshielded carbons and two carbonyl groups detected at δ_C 183.00 and 183.44, respectively.

1-hydroxy-4-methyl anthraquinone (CTREH-01A); appearance: Orange needles (hexane); m p: 349.05 350.00°C; Chemical formula: C15H10O3; molecular weight: 238.23 g/mol; UV (CHCl3) λmax (log ε – molar absorption coefficient): 256.9, 329.2, 411.5; HPLC-Elution time (H2O: Acetonitrile) 70:30 – 22.136; mass spectral data: m/z – 62.90, 152.10, 209.10, 237.10, 238.10, 239.10, 250.10 and 306.10.

DISCUSSION

In our study we used preparative TLC to separate the individual anthraquinones and then subjected the semi-purified extracts to liquid chromatography coupled to a mass spectrometer. The HPLC method was identical to the method employed by Peng (1997) in that both methods employed a C_{18} stationary phase based system. The chromatographic separation for our study utilized by waters Xbridge C_{18} column (150 x 2.1 mm, 3.5 µm) maintained at 40°C. A similar method was also used by Lachâtre (2000) which consisted of symmetry C_{18}, 3.5 µm reverse phase column (waters). The C_{18} stationary phase, mobile phase and mode of detection are comparable to other published HPLC methods for the detection of differently substituted anthraquinones. Three anthraquinone derivatives: (i) 9, 10 anthracenedione, (ii) 1-hydroxy-4-methylanthraquinone, (iii) 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4, 9-dione and a steroid, androst-5-ene-3, 17, 19-triol were isolated from the roots of *C. triloba*. These compounds were verified by analysis of spectral data (UV, ^1H NMR, ^{13}C NMR and EI-LC-MS). The structural similarities of the compounds to mitoxantrone, doxorubicin, daunorubicin, showed that the anthracenedione ring structure is a predominant component of isolated compound in this study that is 9, 10 anthracenedione and 1-hydroxy-4-methylanthraquinone.

Furthermore, the NMR spectral data for 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone showed good correlation to studies by Marques et al. (2000) and Permana et al. (1999). These studies showed the presence of four sets of overlapping multiplets integrating two protons between 7.7-7.8 and 8.2-8.3, indicating a typical aromatic ring which is the structural backbone of the isolated anthraquinones. Data varied for the novel compounds isolated by Marques et al. (2000) and Permana et al. (1999) due to the presence of differently substituted side chains.

Conclusion

Currently, no phytochemical profile for *C. triloba* exists. This research focused on the isolation and structural elucidation of anthraquinone and steroidal derivatives from *C. triloba.* Three anthraquinone derivatives and one steroid were isolated. The structures of these compounds as 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-

tetrahydro-1H-phenanthrene-4,9-dione and androst-5-ene-3,17,19-triol were determined by analysis of spectral data (UV, ^{1}H NMr, ^{13}C NMR and EI-LC-MS). These anthraquinones are similar in structure to mitoxanthrone which is currently the drug of choice for prostate cancer, acute myelogenous leukemia (AML) and breast cancer**.**

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