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PHYSIOCHEMICAL SCREENING OF MARKER ANTHRAQUINONES AND DERIVATIVES FROM THE PENTAS LANCEOLATA (FORSS. K.) DEFLERS LEAVES

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ABSTRACT

Pentas lanceolata (Forssk.) Deflers belonging to family **Rubiaceae,** is a most common plant widely used names are **"Egyptian Star Cluster"** or **"Pentas"**. As a decorative plant, it has been spread all over the tropical and subtropical regions. The Preliminary Phytochemical screening with different solvent extract like Methanol, Chloroform and water with the Leaves of ***Pentas lanceolata*** plant showed Presence of glycosides, sterols, flavonoids, triterpinoids, alkaloids, carbohydrates and resins. Chromatographic separation of the methanolic extract yielded nine **anthraquinones** and derivatives, of which **Rubiadin-1-methyl ether-3-*O*-β-primeveroside**, **Rubiadin-1-methyl Ether**, **Damnacanthol (3-hydroxy-2-hydroxymethyl-1-methoxy-9, 10-anthraquinone)**, **Rubiadin (1,3-dihydroxy-2-methyl-9,10- anthraquinone)** and **Lucidin-ω-methyl ether (1,3-Dihydroxy-2-methoxymethyl-9,10-anthraquinone)** were separate out by Preparative TLC and screened out by different spectroscopic techniques. The one steroidal compound was also isolated and was identified as **(24*S*)-24-Ethylcholesta-5-(*E*)-22-dien-3β-ol (β-Stigmasterol)**.

KEYWORDS: Methanol, Anthraquinones, Sterols, Preparative TLC, NMR, β-Stigmasterol.

INTRODUCTION

Pentas lanceolata, commonly known as Egyptian Star cluster, is a species of flowering plant belongs to family, **Rubiaceae** that is native to much of Africa. It is known for its wide use as a garden plant where it often accompanies butterfly gardens. *P. Lanceolata* is evergreen shrub or tall perennial becomes **3 to 4 feet** tall and is decorated throughout most of the year in hardiness zones 9 through 11 with many 3-inch-wide, dense clusters of long-tubed, **star-shaped flowers** Available in white, pink, red and lavender, these blossoms are extremely popular with butterflies and are long lasting as cut flowers. Plants **fertilized** regularly during the growing season will continue to grow and bloom all during the warm months. **Leaves and stems** are covered with fine hairs, and **leaves** have prominent veins on the undersides.[1] In Kenya, a decoction of its roots mixed with milk is takes as a cure for malaria.[2]

Plant Profile[3]

Pentas lanceolata* (Forssk.) Deflers** belonging to family ***Rubiaceae that is commonly known as Egyptian Starcluster, Pentas, **Starflower** etc.

Syn: - *Pentas lanceolata*, and *Pentas longiflora* etc.

Classification: - Kingdom: - Plantae – Plants
Subkingdom: - Tracheobionta – Vascular plants
Supervision: - Spermatophyta – Seed plants
Division: - Mangoliophyta – Dicotledons
Subclass: - Asteridae
Order: - Rubiales

Family: - **Rubiaceae**

In a phytochemical investigation of ***P. bussei* root** was published.[4] In this study, β -stigmasterol and the anthraquinones damnacanthol, rubiadin-1-methyl ether, rubiadin, 1,3-dihydroxy-2-methoxymethyl-9,10-anthraquinone, damnacanthol- 3-O-methyl ether and rubiadin-1-methyl ether-3-O--primeveroside were isolated.

In the present study, we isolated and identified six new anthraquinones and derivative from the leaves of *P. lanceolata* and their structural characterization is described herein.

By using a ^{1}H and ^{13}C NMR-based screening procedure, specific signals indicating the presence of unknown compounds were observed for a methanolic extract of the leaves of ***P. lanceolata*.** This urged us to proceed with the isolation and identification of these new anthraquinines.

MATERIAL AND METHODS

Plant Material

The leaves of ***Pentas lanceolata*** (Forssk.) Deflers was required for the extraction and isolation of Anthraquinones and derivatives was collected from Kanpur Dehat, Uttar Pradesh east regions and was authenticated by the Botany Department of Rajasthan University, Jaipur was dried and Pulverised in coarsely powder form. (Authentication specimen voucher No.RUBL30856).

Solvent Used

Methanol, Chloroform and water were used for extraction purpose. All the solvent were analytical grade and procured from CDH Pvt. Ltd.

Extraction method

The leaves of *Pentas lanceolata* were subjected to fine powder and 300gms for each were subjected to continuous successive extraction with different solvents like Methanol, Chloroform and Water in soxhlet extractor. After complete extraction, the different solvents (Methanol, Chloroform and Water) were concentrated to water bath and finally dried under reduced pressure to the dryness in flash evaporator. After drying the respective extracts were weighed and calculated percentage yield.

Screening Methods

A) Chemical identification

After complete extraction of the plant material, the filtration and concentration of the extract was done under reduced pressure and percentage yield was calculated for each of the extract. Concentrated extracts were subjected for preliminary phytochemical identification of the major class of compounds like alkaloids, glycosides, terpenoids, tannins and Resins etc as per standard procedures.[5,6]

B) Separation and Purification

Thin layer chromatography was on Merck thin layer chromatography (TLC) F254 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 immediately to minimize the possibility of oxidative or photo-oxidative change. Separated components were visualized under visible and ultraviolet light. Plates were also sprayed with p-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.[7,8]

Column chromatography

Silica gel 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. The column was left to run overnight at a flow rate of 0.5 ml/min.[9]

C) Screening of the separated fractions

Different 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 F254) starting and ending at least 1 cm from either side. The plate was developed in the mobile phase and the bands visualized under ultraviolet light (254 and 360 nm).

D) *IR, ^{1}H NMR and C^{13} NMR spectroscopy*

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 mode. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR. The $CDCl_3$ was used as the solvent of choice. The samples were then pipette into NMR tubes with the aid of a Pasteur pipette. ^{1}H NMR was run at 400 MHz and ^{13}Cat 75 MHz using the solvent signal tetra methyl silane

(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.[10]

E) Preparative high performance liquid chromatography and uv-vis spectroscopy of compounds isolated from *Pentas lanceolata*

Partially purified extracts/fractions were coded as **PLMeEx01, PLMeEx02, PLMeEx03, PLMeEx04** and **PLMeEx05** and further purified using preparative HPLC. **Figures 3, 4 and 5** illustrate the separation of pure compounds from **PLMeEx01, PLMeEx03** and **PLMeEx05** respectively. The compounds that were isolated from Preparative TLC were not pure enough to determine their structures using LC-MS. All three extracts contained similar compounds at retention times of **22.133** and **4.5 min.**

RESULTS

The concentrated extracts was obtained as yellowish-green ***Methanol*** residue (26.07 gm, 8.69% yield), a black green **Chloroform** residue (8.69 gm, 2.89% yield) and a greenish **water** residue (5.87 gm, 1.95% yield) was calculated.

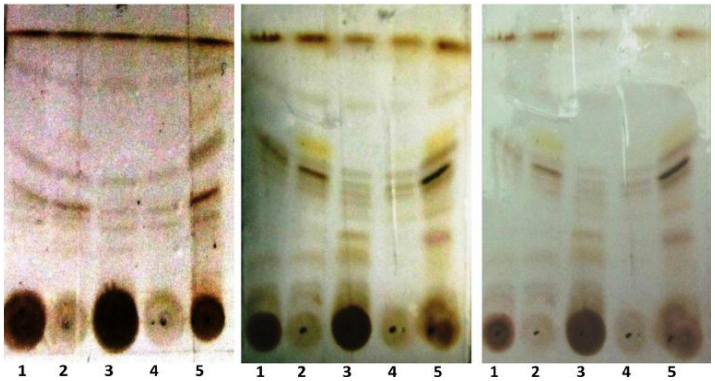
Preliminary Phytochemical Screening with the crude extracts of leaves showed the presence of **Anthraquinone Glycosides**, Terpenoids and Lipids as major fractions in all three extracts. No flavonoids, tannins and cardiac glycosides were detected. These results are shown in **Table 1.**

Table1.

Sl. No.	Drug extract → / Test for ↓	Methanol extract	Chloroform extract	Water extract
1.	Carbohydrates	-	-	+
2.	Protein/amino acid	-	+	-
3.	Alkaloids	+	-	-
4.	Glycosides	+	+	+
5.	Flavanoids	-	-	+
6.	Tannins	-	-	-
7.	Phytosterol	-	+	-
8.	Terpenoids\volatile oils	-	+	+
9.	fats and lipids	+	+	-

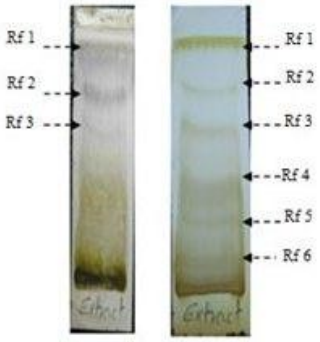
TLC Profile

By using optimized separation and detection parameters, the components in the different portals of the plant were compared. A profiling of the **Rf values** from leaves showed five common compounds at **Rf values** of **0.21, 0.34, 0.38, 0.41 and 0.39. (Figure1-2).**



Methanol Extract **$CHCl_3$ Extract** **Water Extract**

Fig. 1



Methanol Extract

Fig. 2

Preparative HPLC of the Fractions: All three extracts contained similar compounds at retention times of **22.133** and **4.5 min. (Figure3-5).**

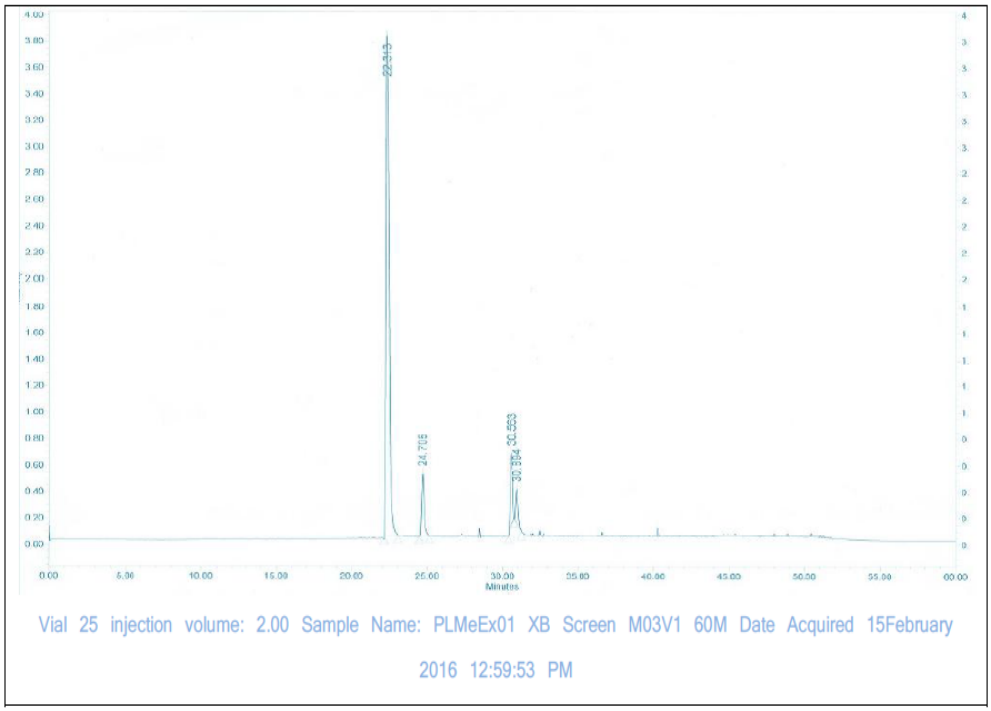


Figure3. Preparative High Performance Liquid Chromatography (P-HPLC) of PLMeEx01 showing the presence of four distinct compounds at varying retention times. A-Retention time = 22.136, B- Rt = 24.705, C- Rt = 30.563 and F- Rt=30.894.

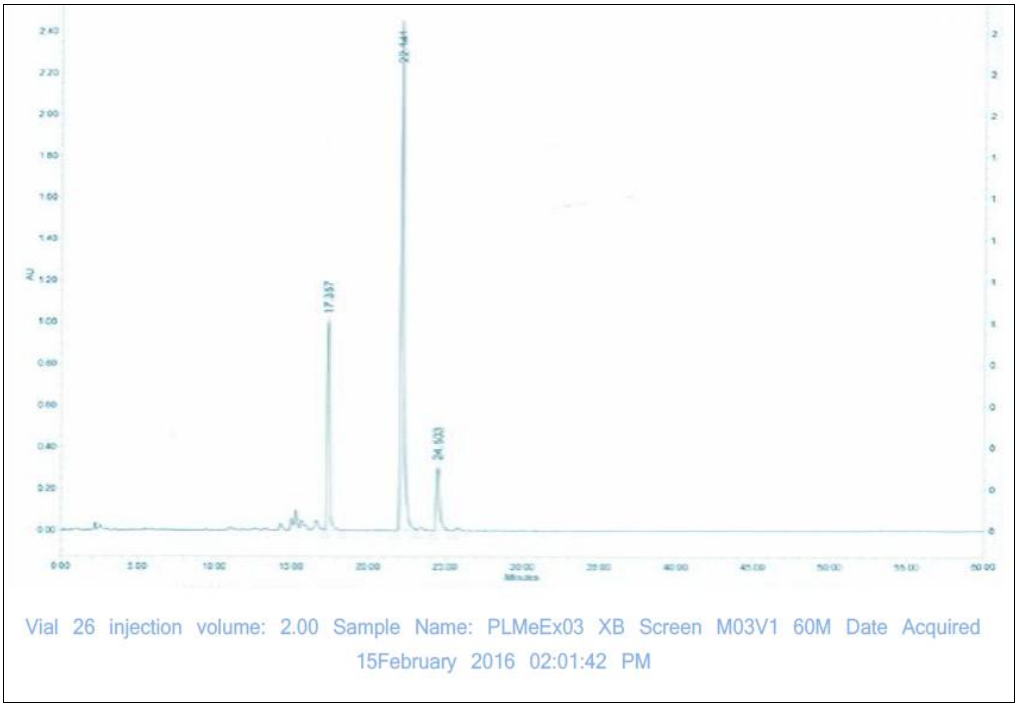


Figure4. Preparative High Performance Liquid Chromatography of PLMeEx03 showing the presence of three distinct compounds at varying retention times. D-Retention time = 17.357, A- Rt = 22.141 and B- Rt = 24.503.

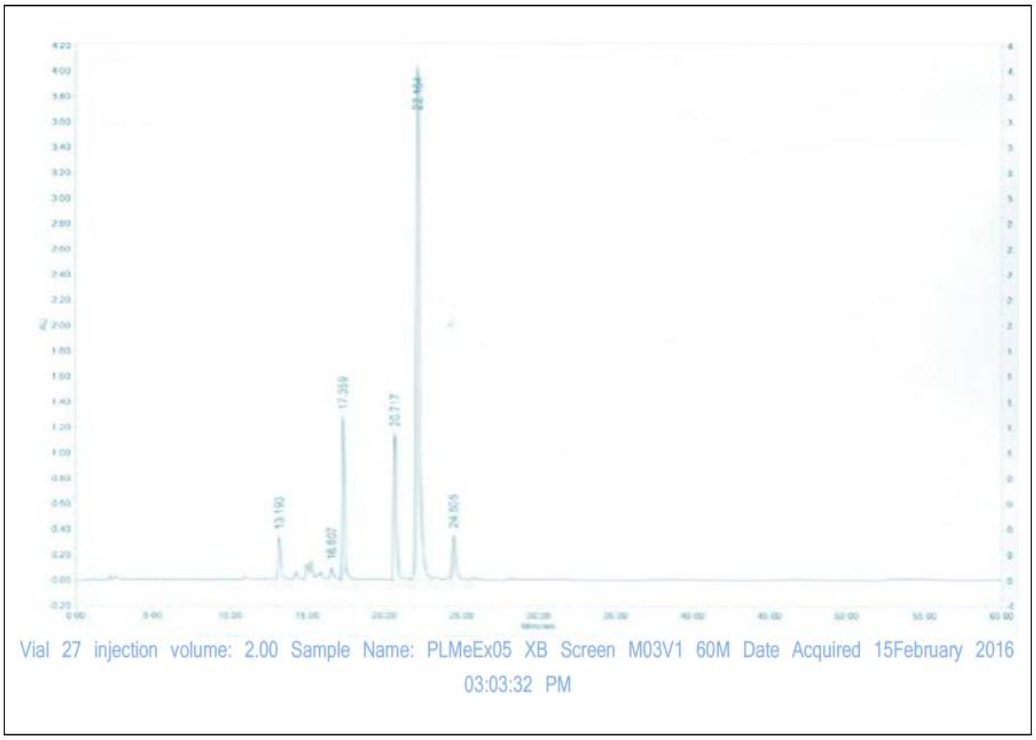


Figure5. Preparative High Performance Liquid Chromatography of CTREh03 showing the presence of four distinct compounds at varying retention times. D-Retention time = 17.359, E- Rt = 20.717, A- Rt = 22.164 and B-Rt = 24.505.

Liquid LC-MS analysis of purified components of *P. lanceolata*

LC-MS indicated six different compounds in **all three active portions** of the extract with varying structural differences representing anthraquinones and one Steroid. Prevalent compounds belonged to the anthraquinones which included **Rubiadin-1-methyl ether-3-*O*-β-primeveroside** (Figure 6), **Rubiadin-1-methyl Ether** (Figure 7), **damnacanthol (3-hydroxy-2-hydroxymethyl-1-methoxy-9, 10-anthraquinone)** (Figure 8), **Rubiadin (1,3-dihydroxy-2-methyl-9,10-anthraquinone)** (Figure 9) and **Lucidin-ω-methyl ether (1,3-Dihydroxy-2-methoxymethyl-9,10-anthraquinone)** (Figure 10). The steroid isolated was identified as **(24*S*)-24-Ethylcholesta-5-(*E*)-22-dien-3β-ol (β-Stigmasterol)** (Figure 11).

Identification of the Compounds

Identification of Rubiadin-1-methyl ether-3-*O*-β-primeveroside: - Dark yellow colour amorphous Powder; KBr, cm^{-1}), 3342, 2984, 2911, 1736, 1372, 1043 cm^{-1}; ^{1}H NMR (CDCl3, 500 MHz), δ 8.82 to 8.80 (2H, d, Ar - protons H-6,7), δ 7.88 to 7.86 (2H, d, Ar-proton, H-5,8), δ 6.15 (^{1}H, d, Ar - proton, H-4), δ 5.81 (1H, m, H-1'), 5.03 (1H, m, H-1"), δ 3.83 (3H, s, OCH3 group,) δ 3.71 to 3.01 (9H, m, CH protons of pyranose ring), δ 2.61 (3H, s, CH3 group). ^{13}C NMR (CDCl3, 125 MHz): Table 1; $C_{29}H_{30}O_{14}$; EIMS m/z: 578$[M]^+$, 601$[M+Na]^+$.

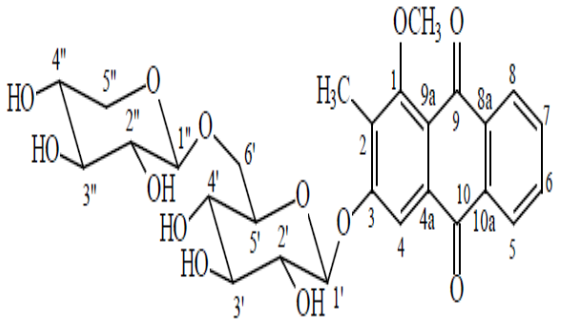


Figure: - 6 - Rubiadin-1-methyl ether-3-*O*-β-primeveroside

Identification of Rubiadin-1-methyl Ether

Yellow crystals; mp 289.3-291.2°C, lit. mp 292-294°C (146a). The IR spectrum of compound displayed an absorption band of a phenolic hydroxy group at 3304 cm^{-1}. The spectrum also showed bands of the carbonyl groups at 1673 and 1651 cm^{-1}, respectively. The **^{1}H NMR** spectrum of the compound in DMSO-d6 displayed signals of a methyl group at δH 3.79 (3H, s, OCH3-1), whereas a singlet at δH 7.50 (1H, s, H-4) and an AA'BB' coupling system of four protons at **δH 7.83-7.92 (2H, m, H-5 and H-8)** were observed in the aromatic region. The latter system indicated that all the substituents were on one aryl ring. The **^{13}C NMR** spectrum showed sixteen signals from which those of the two carbonyl carbons at δC 180.10 and 182.51 ppm. In addition, two =Cq-O

signals were observed at δC 160.50 and 161.60 ppm, whereas the methoxy carbon resonated at **δC 60.48 (OCH3-1).** Such a high δC value for the methoxy carbon is consistent with its *peri* location (1-position) from the carbonyl in 9-position, as observed in several compounds with the same configuration. Based on biogenetic grounds, the methyl group may be located in the 2-position. The EI mass spectrum of the compound displayed an ion peak **at *m/z* 268 (6%)** which is consistent with the molecular formula of **$C_{16}H_{12}O_4$.**

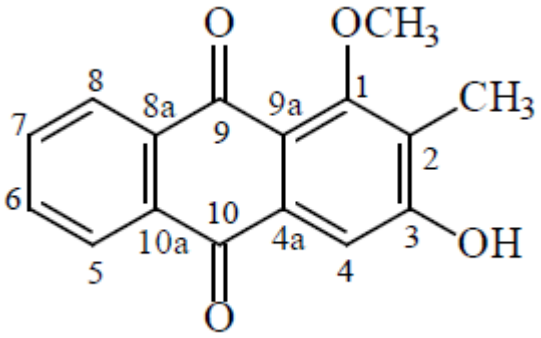


Figure: - 7 - Rubiadin-1-methyl Ether

Identification of damnacanthol (3-hydroxy-2-hydroxymethyl-1-methoxy-9, 10-anthraquinone)
Yellow solid, mp 284.8-287.1°C. The UV spectrum of the compound was compatible with an anthraquinone structure by showing absorption bands at 239.0, 249.0 and 280.0 nm. The IR spectrum showed absorption bands of **OH groups at 3308 cm^{-1}**, together with bands of carbonyl groups at 1673 and 1651 cm^{-1}. From analysis of **the ^{1}H, and ^{13}C NMR** spectra were disclosed the Methoxy group at δ_H 3.86 (3H, s, OCH3-1)/ δ_C 62.26 (OCH3-1) and the hydroxymethylene group at **δ_H 4.57 (2H, s, CH2OH-2)/ δ_C 52.4 (CH2OH-2)** and a sp^2 CH at δ_H 7.49 (1H, s, H-4)/ δ_C 109.93 (C-4). Furthermore, no influence of the hydrogen bond was observed, neither in the IR spectrum, nor in the ^{13}C NMR δ_C values of the two carbonyl carbons **($\Delta\delta_C$ 2.76 ppm).** The ^{1}H NMR spectra showed a four proton AA'BB' coupling system corresponding to the four adjacent aryl protons. The **^{13}C NMR spectrum** showed sixteen signals. Two carbonyl carbons were observed at δ_C 179.82 and 182.58 ppm. whereas two resonated at δ_C 161.54 and 162.75 ppm, respectively. The mass spectrum displayed ions at *m/z* 307 (80%), 285 (45%) and 267 (100%), corresponding to the fragments **$[M + Na]^+$, $[M + H]^+$ and $[M – H2O + H]^+$,** respectively. From this pattern was deduced a molecular formula of $C_{16}H_{12}O_5$ for the compound as damnacanthol (3-hydroxy-2-hydroxymethyl-1-methoxy-9,10-anthraquinone).

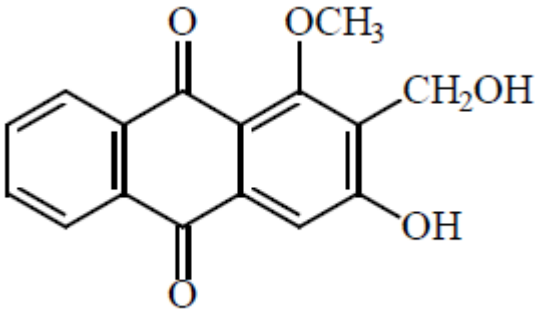


Figure: - 8 – Damnacanthol

Identification of Rubiadin (1,3-dihydroxy-2-methyl-9,10- anthraquinone)
Yellow solid; mp 298.8-302.4°C. The UV spectrum of the compound displayed a pattern characteristic of an anthraquinone with two absorption bands at **245.9, 281.0 and 412.0 nm.** The IR spectrum showed an absorption band of phenolic hydroxy groups at 3391 cm^{-1}, together with both non-hydrogen and hydrogen bonded carbonyl groups at 1662 and 1624 cm^{-1}, respectively.

The **^{1}H NMR spectrum** of the compound displayed a low-field OH proton signal at δ_H 13.10 ppm, whereas the ^{13}C NMR spectrum showed that the chemical shifts of the two carbonyl carbons were largely separated at **δ_C 181.65 (C-10) and δ_C 186.00 ppm (C-9)** with a $\Delta\delta_C$ value of 4.35 ppm. Therefore, a hydroxy group is unequivocally located in the *peri* position from carbonyl in 9-position and this is consistent with the chelation observed in the IR spectrum. In addition, the NMR spectra (**^{1}H NMR, ^{13}C NMR and HETCOR**) showed one methyl at δ_H 2.05 **(1H, s, CH3-2)/ δ_C 7.94 (CH3-2), a =CH at δ_H 7.2 (1H, s, H-4)/ δ_C 107.28 (C-4), together with two =Cq-O at δ_C 162.31 and 162.80 ppm**. The ^{1}H NMR particularly disclosed a four protons AA'BB' coupling system at δ_H 7.89 (2H, m, H-6 and H-7) and 8.18 (2H, m, H-5 and H-8), which is consistent with the location of the aromatic proton H-4, the two hydroxy groups and the methyl group on one aryl ring.

From all the above presented data, the structure of the compound was elucidated as being the known **anthraquinone rubiadin** (1,3-dihydroxy-2-methyl-9,10-anthraquinone).

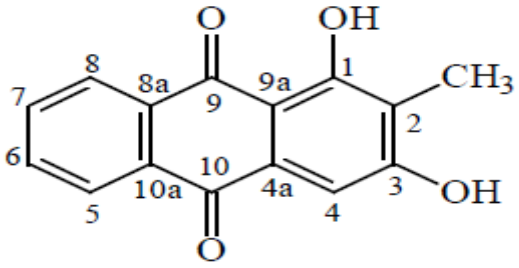


Figure: - 9 - Rubiadin (1,3-dihydroxy-2-methyl-9,10-anthraquinone).

Identification of Lucidin-ω-methyl ether

Amorphous yellow powder; mp 170.8-172.4°C. The UV spectrum of the compound showed absorption bands at 241.0, 247.0 and 281.0 nm which are consistent with an anthraquinone structure. In the IR spectrum, the absorption bands of the OH groups were observed at **3394 and 3197 cm^{-1}.** The absorption of carbonyl groups were observed at 1674 cm^{-1} (unchelated C=O) and 1627 cm-1 (chelated C=O), whereas a C-O-C group absorbed at 1275 cm^{-1}.

The **^{1}H NMR spectrum (in $CDCl_3$)** displayed a hydroxy proton signal in low-field at δ_H 13.31 (1H, s, OH-1), obviously corresponding to the OH-1 in *peri* position from the carbonyl oxygen in 9-position. This OH-1 was associated with the hydrogen bonding observed in the IR spectrum. An additional hydroxy group was observed at **δ_H 9.43 (1H, s, OH-3)**. Besides, the aromatic proton signals were typical of an anthraquinone having a non-substituted and a trisubstituted aryl ring by showing a symmetrical AA'BB' coupling system of four protons **at δ_H 7.77-7.79 (2H, m, H-6 and H-8) and δH 8.25-8.29 (2H, m, H-5 and H-8),** together with an aromatic proton singlet at δH 7.32 (1H, s, H-4). The methoxy and the oxymethylene protons were observed at 3.58 and 4.94 ppm respectively.

From the conjugation and the hydrogen bonding effect of the OH-1, the ^{13}C NMR spectrum showed largely different chemical shifts for the two carbonyl carbons at **δ_C 182.23 (C-10)** and **186.88 (C-9).** The two =Cq-O were observed at δ_C 161.88 and 164.08 ppm. The location of the methoxy group in 3-postion was ruled out by the presence of the OH-3 proton signal at δ_H 9.43 in the ^{1}H NMR spectrum. Moreover, the strongest evidence of the benzylalkyl ether nature (rather than an arylalkyl one) for compound came from the ^{13}C NMR spectrum which showed the chemical shifts **values δ^C 68.89 (CH_2OCH_3-2) and δ_C 59.35 (CH_2OCH_3-2).** These values are typical of ethers for which the two alkoxy groups give rise to a reciprocal shift to down-field of more than 10 ppm in ^{13}C NMR, relatively to the corresponding alcohols. The two carbons exert to each other a reciprocal influence similar to that of a C-β in alkanes, the oxygen atom being considered as **a C-α. The fragment [M + Na]$^+$ was observed at *m/z* 307 (14%)** in the ESI mass spectrum. Therefore, the molecular formula was deduced as being $C_{16}H_{12}O_5$. On the basis of all the above discussed spectroscopic data, the structure of the compound was established as **lucidin-ω-methyl ether (1,3-dihydroxy-2-methoxymethyl-9,10-anthraquinone).**

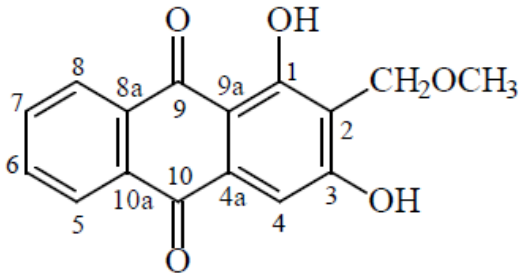


Figure: - 10 - lucidin-ω-methyl ether

Identification of β-Stigmasterol ((24*S*)-24-Ethylcholesta-5-(*E*)-22-dien-3β-ol)

White solid, mp 165.0-166.9 °C. The IR spectrum of the compound showed the free OH group at 3406 cm^{-1}. The EI mass spectrum displayed a molecular ion peak **M^+ at *m/z* 412 (3%)** which is compatible with the molecular composition of $C_{29}H_{48}O$ with an unsaturation index of 6.

The ^{1}H NMR spectrum disclosed two signals which are typical of steroidal compounds at δ_H **3.46-3.61 (1H, m, H-3)** ascribed to the proton of the methine bearing the OH group and at δ_H **5.35-5.38 (1H, m, H-6)** attributed to the olefinic proton in the ring B. In addition, a particular AB coupling system with a Δν of 62.7 Hz was observed at 4.96-5.18 (2H, qd, ^2J = 15.2 Hz, ^3J = 8.7 Hz and 8.4 Hz, H-22 and H-23) and was consistent with the presence of an olefinic unit CH=CH in the side chain.

Analysis of the ^{1}H NMR spectra also indicated the presence of three methyl groups having their protons resonating as doublet at **δ_H 0.77 (3H, d, *J* = 6.4 Hz, CH3), 0.84 (3H, d, *J* = 6.2 Hz, CH3) and δ_H 1.04 (3H, d, *J* = 6.8 Hz, CH3)**, together with another one with protons resonating as triplet at δ_H 0.81 (3H, d, *J* = 6.4 Hz, CH3).

Furthermore, the ^{13}C NMR spectra displayed signals for six methyl groups, nine sp3 methylene groups, eight sp3 methine groups, three sp2 methine groups, two sp3 quaternary carbons and one sp2 quaternary carbon.

Comparison of all the above mentioned data with those of various steroidal compounds led to the identification of the compound as **(24*S*)-24-ethylcholesta-5-(*E*)-22-dien-3β-ol (β- stigmasterol)**.

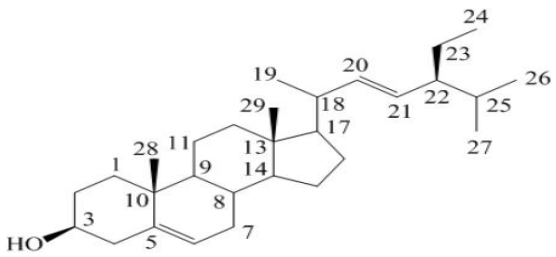


Figure: - 11 - β-Stigmasterol ((24*S*)-24-Ethylcholesta-5-(*E*)-22-dien-3β-ol)

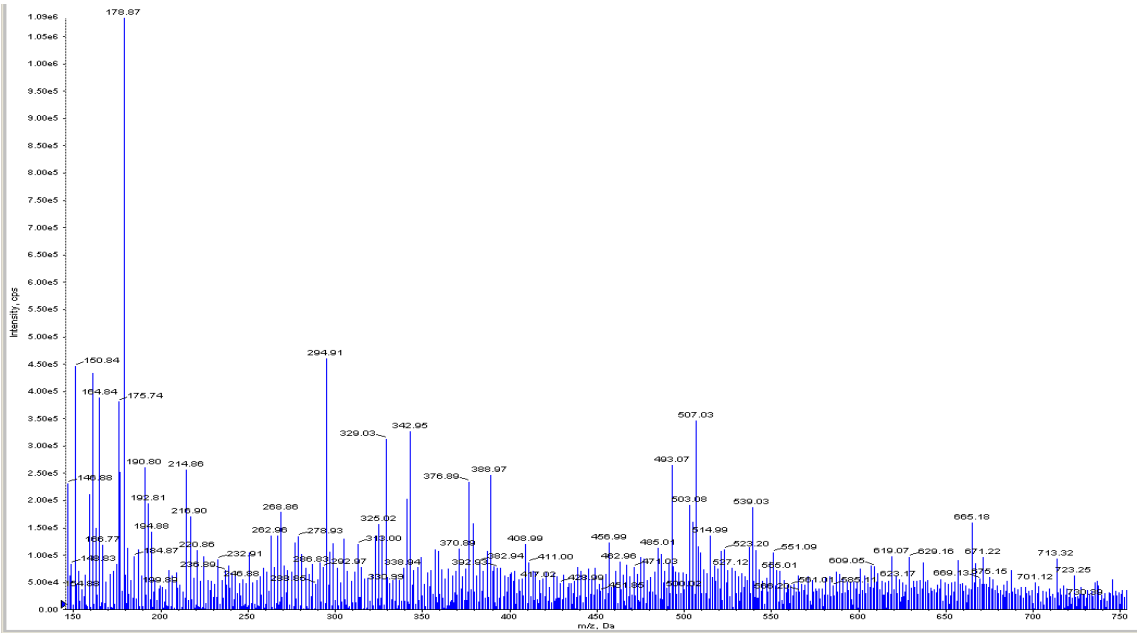


Figure: - 12 - Total Mass Spectrum

CONCLUSION

The fractionation by various chromatographic methods of the **Methanol extracts** of the leaves of ***P. lanceolata*** resulted in the isolation of the five anthraquinones as **Rubiadin-1-methyl ether-3-*O*-β-primeveroside** (Figure 6), **Rubiadin-1-methyl Ether** (Figure 7), **damnacanthol (3-hydroxy-2-hydroxymethyl-1-methoxy-9, 10-anthraquinone)** (Figure 8), **Rubiadin (1,3-dihydroxy-2-methyl-9,10- anthraquinone)** (Figure 9) and **Lucidin-ω-methyl ether (1,3-Dihydroxy-2-methoxymethyl-9,10-anthraquinone)** (Figure 10). The methanol extract also contains sufficient quantity of a steroid isolated and identified as **(24*S*)-24-Ethylcholesta-5-(*E*)-22-dien-3β-ol (β-Stigmasterol)** (Figure 11).

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