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ORIGINAL ARTICLE, MEDICINE

Triterpenoid and Fatty Acid Contents from the Stem Bark of *Cordia dichotoma* (Forst f.)

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Aim: To isolate and determine the chemical constituents of the stem bark of *Cordia dichotoma* (Forst f.), a plant used for medicinal purpose in folk medicine.

Materials and methods: Petroleum ether extract of the stem bark was used for this study. Saponification process was performed to separate fatty acid and unsaponifiable matter.

Results: One triterpenoids, α-amyrin was isolated from the bark by using isocratic elution. The chemical compounds isolated, for the first time, were analyzed by GC/MS, IR, and UV. The chemical composition of the fatty acids methyl esters (FAMEs) in bark of *Cordia dichotoma* were also analyzed by gas chromatography-mass spectrometry. After methyl-esterification, 17 components were identified in the bark. The derivatization conditions were investigated in order to validate this method.

Conclusion: The present analysis revealed that *Cordia dichotoma* stem bark contains 17 fatty acid. The principal themes of the review highlight the development and application of chromatographic techniques for the separation, isolation and detection of the compounds.

BACKGROUND

The lipids are a heterogeneous group comprising mainly substances with low polarity and limited water solubility. They are often defined by their special solubility properties. Lipids can be extracted from living tissues with alcohol or ether.[1] The term 'lipids' embraces a variety of chemical substances including fixed oils, fats, waxes, phosphatides and lecithins. These substances are widely distributed in nature both in vegetable and animal kingdoms and are of great pharmaceutical importance. In recent years considerable advances have been made in refining the methods available for analyzing the lipid constituents of plants. Several excellent reviews are available that highlight the merits and drawbacks of different procedures.[2-6] Details about the classical methods of extraction and isolation of lipids can be found from the reviews reported by Hilditch and Williams[7], James and Morries[8], and Kaufmann[9] where they have described the methods for extraction of polar lipids. Flotch and co-workers have described a method for initial isolation of lipids which is quite popular.[10] Another extensively employed procedure has been described by Bligh and Dyer.[11]

For fractionation of separation and purification of different types of components, different chromatographic techniques like paper, column, thin layer and gas chromatography have become indispensable. Although many techniques have been developed for the fatty acids analysis, gas chromatography with mass spectroscopy (GC-MS) is still the most widely used one. This well-established procedure is very efficient and rapid when complex mixture with a broad range of molecular weights has to be analyzed.

However, the inherent low volatility of fatty acids has been a problem. Two main approaches adopted for the examination of analytes do not seem to satisfy the normal criteria of volatility for GC-MS. Either is degraded under controlled conditions by pyrolysis, to give characteristic volatile fragments or they are derived into related compounds that are suitable for gas chromatography. Consequently, they are converted into more volatile derivatives (such as fatty acid methyl esters) when they are analyzed by gas chromatography (GC).

Although many of the lipids and the fatty acids methyl esters (FAMEs) of various plants have been extensively investigated to obtain volatile components, the need still remains for unstudied plants.

Cordia dichotoma (Forst f.) is a plant belonging to the boraginaceous family and is widely distributed in India; it is commonly known as *Lasura* in Hindi and *Shlesmataka* in Sanskrit. It is used as an immunomodulator, antidiabetic, anthelmintic, diuretic and hepatoprotective in folk medicine. Seeds have disclosed the presence of α–Amyrin, betulin, octacosanol, lupeol–3–rhamnoside, β–sitosterol, β–sitosterol–3–glucoside, hentricontanol, hentricontanol, taxifolin–3,5–dirhmnoside, and hesperitin–7–rhamnoside.[12-14]

Preliminary phytochemical analysis of *C. dichotoma* stem bark indicated the presence of relatively high levels of steroids, terpenoids, alkaloids and flavonoides.[15] No accurate determination of the lipid components in *C. dichotoma* bark has been reported so far. Hence, the present investigation was undertaken to determine the chemical potential of saponifiable and unsaponifiable matter in *C. dichotoma* stem bark.

MATERIALS AND METHODS

Chemicals and reagents

Petroleum ether (60°-80°), methanol, KOH, H_2SO_4, anhydrous Na_2SO_4, n-hexane, diethyl ether, glacial acetic acid, benzene, silica gel (60-120° mesh size) were purchased from Meark (India).

Plant materials

Fresh stem bark of *Cordia dichotoma* was collected from Jamnagar (Gujarat), India in 2009 and was authenticated by Department of Pharmacognosy of Gujarat Ayurved University, Jamnagar, India.

Preparation of extracts

The bark was shade dried and crushed to make coarse powder. The powder was extracted with petroleum ether (60°-80°) by continuous Soxhlet's extraction method for 48 h. The solvent was distilled off and the extract was concentrated and dried under reduced pressure, which yielded a brownish mass. This crude extract was used for further investigation.

Experimental[16]

An accurately weighed pet ether (1.05 gm) was taken in flask and 50 ml of 10% methanolic KOH was added and kept overnight at room temperature. On the next day, the mixture was refluxed for 6 hrs and cooled at room temperature. Twice volume of distilled water was added and extracted with ether. Combined ethereal extract was washed with distilled water till it became neutral to litmus paper and dried over anhydrous sodium sulphate. Afterward, the ether was evaporated to obtained unsaponifiable fraction (27 mg) (fraction A).

The aqueous portion left after ether extraction was named as saponifiable fraction. It was extracted and acidified with 5N H_2SO_4 and the aqueous layer was extracted 3-4 times with ether. Ethereal layer was washed with distilled water and extractive was dried over anhydrous Na_2SO_4, evaporated to obtain fatty acid portion (0.292 gm) (fraction-B).

Investigation of unsaponifiable fraction: (fraction A)

The unsaponifiable fraction (fraction A) was tested with Liebermann–Burchard reagent for the confirmation of steroid and triterpenoids which gave a strong positive test. The unsaponifiable fraction (27 mg) was concentrated, dried and subjected to column for separation, column chromatography was carried out in long narrow column on Kieselgel silica (60-120-mesh) (Merck). A fritted-glass disk may be seated in the end of the tube to act as a support for the packing material. A small fraction of dried sample was chromatographed on 30-50 times of its volume of silica gel 60-120° mesh size and prepared column. Column was build-up with hexane. Isolation of compound achieved using isocratic elution, with solvent system n-hexane: diethyl ether: GAA (glacial acetic acid) (7:3:0.5). Obtained fractions were monitored on thin layer chromatography and were analysed by IR, UV and GC-MS.

Investigation of saponifiable fraction: (fraction B)

Methylation of fatty acid

An accurately (94 mg) weighed portion of (fraction B) fatty acid was taken into flask and refluxed in water bath for 8 hrs with solution of absolute

methanol: benzene: Conc. H_2SO_4 (43:5:2). Cooled the same and diluted with (40 ml) water and extracted using hexane. The extract was dried over $NaHCO_3$:Na_2SO_4 (1:3) and volume was made up to 10 ml with hexane and stored for further GC-MS analysis.

Instruments

FT-IR analysis

FTIR analyses were carried out on IR-200 Thermo Nicolet series FTIR instrument. Samples were ground with spectroscopy grade KBR and a pellet of homogeneous mixture was prepared using hydraulic press. Care was taken for the maximum opacity of the pellet and IR spectra was recorded between 4000 cm^{-1} to 400 cm^{-1}.

UV/Visible analysis

A Systronics λ-2 double beam UV/Visible spectrophotometer-2201 was used for all spectral analyses. The instrument was preloaded with software and the spectra were recorded in the range of 190-700 nm with background correction. Direct isolated fraction was applied to UV.

GC-MS protocol

GC-MS analysis was carried out on a Shimadzu GC-MS model no. QP 2010. sampler and gas chromatograph interfaced to a mass spectrometers (GC-MS) instrument employing the following conditions: Column Elite-1 fused silica capillary column (30mm×0.25mm ID ×1 μ M df, composed of 100% Dimethyl poly siloxane), operating in an electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 μl was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 10°C/min, to 220°C, then 5°C/min to 280°C, ending with an isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da.

RESULTS

Interpretation on GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Unsaponifiable (fraction A)

Compound-1: α-Amyrin, M.F.-$C_{30}H_{50}O$, M.W. - 426, RT-20.41

GC-MS fragment

The peak at 20.41 minutes had a mass [M^+] 426. The daughter ion spectra of these compounds (inserts) revealed the characteristic fragments *m/z* 43(100), 60, 73, 143, 185, 218, 343,373,412 and 426 (**Figs 1, 2**).

IR DATA

In IR spectral analysis presence of -OH group (peak at 3479 cm^{-1}), =CH, -CH3, -CH2, (peak at 3340, 2937, 2912, 2848 cm^{-1}, respectively), unsaturation (peak at 1730 and 1670 cm^{-1}) were indicates C=O group, aromatic C=C (peak at 1456 cm^{-1}), 1372 cm^{-1} indicates str. Aromatic C=C , C-H, 1288, 970 cm^{-1} for C=C Aro. Sub, 888-773 cm^{-1}- O-Sub and P-Sub of benzene and 767, 744, 699, 644 cm^{-1} were in plane and out of plane banding (**Fig. 3**).

U.V. Data - Abs at 254 nm and 320 nm indicating extended S-bond system and n-s* transition.

Taking all the above data into consideration, the compound may be equated as Amyrin. It has the following structure fragment. The spectrum also shows M^+ peak-426 indicating molecular weight of the compound is 426. Further fragment indicating compound is Amyrin but correct isomer was not found. But α-Amyrin was reported in *C. dichotoma* seed, hence, the compound may be α-Amyrin.

Saponifiable (identification of fatty acids)

The major fatty acid components were identified by noting their retention time and comparing with retention time of authentic sample of methyl ester of fatty acid. GC analysis of the ester residue showed several peaks (**Fig. 4**). The area (%) covered by individual peak. **Table 1** showed that the 17 compounds were identified in *C. dichotoma* bark. Major FAMEs was identifying like 2-methyl pentane (18.29%), bis-isopropyl (20.79%), 1-nonene, 2-methyl (18.34%), Cicloesano (12.47%) and other identified compounds was small molecule compound (**Table 1**).

DISCUSSION

The results showed in the formation of water soluble alkali salts of fatty acids. The unsaponifiable matter was separated through extraction with non polar solvents like diethyl ether. From the unsaponifiable fraction, one triterpenoid compound was identified

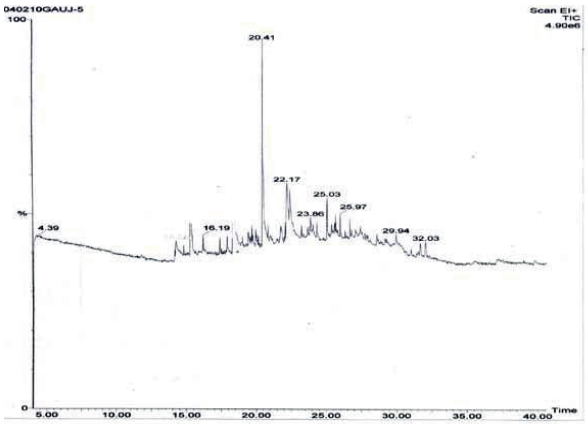


Figure 1. GC graph of α-Amyrin

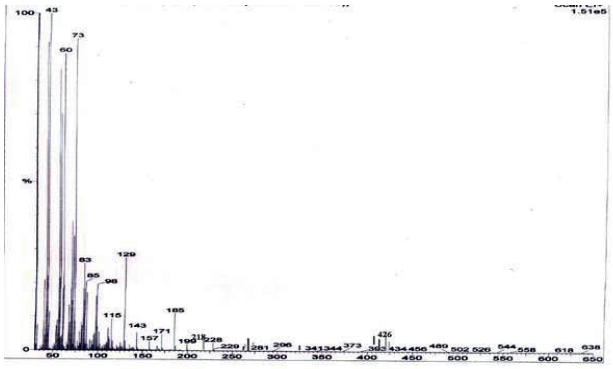


Figure 2. Mass graph of α-Amyrin

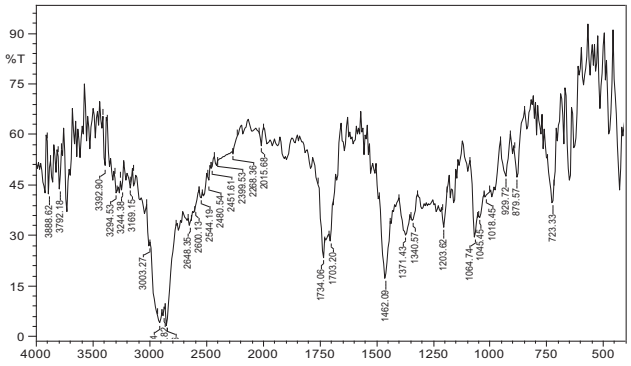


Figure 3. IR graph of α-Amyrin

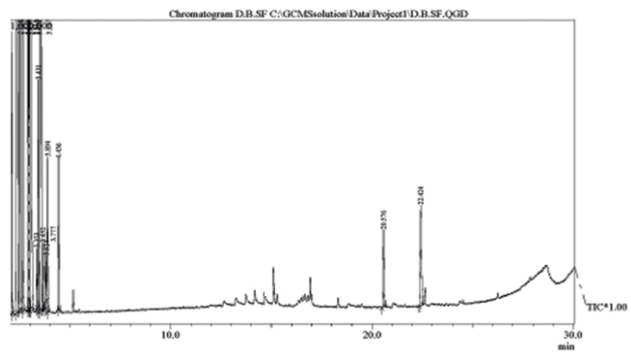
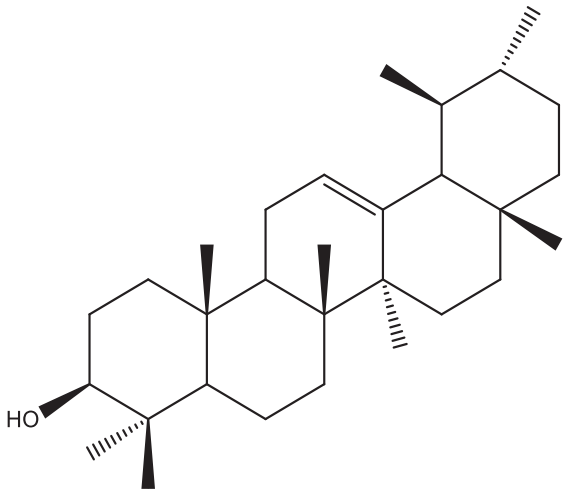


Figure 4. GC chromatogram of methyl ester fatty acid of *C. dichotoma* bark.



Structure of α-Amyrin

and saponifiable fraction; the mixed fatty acids were liberated by addition of 5N sulphuric acid. It was difficult to separate higher fatty acids through simple chemical methods; hence, they were converted into their methyl esters which were amenable to separation through various chromatographic techniques like adsorption TLC, reversed phase TLC and GC-MS. In the present study GC-MS technique has been employed to study the unsaponifiable and saponifiable matter in the test plant. The comparison of the mass spectrums with the data base gave more than 95% match as well as confirmatory compound structure match.

CONCLUSIONS

In the present study, we extracted and identified seventeen fatty acid components in saponifiable fraction and isolate one triterpenoid (α-Amyrin) from unsaponifiable fraction from *Cordia dichotoma* stem bark. It is the first time reporting in this plant. These results indicated the extract is rich in fatty compounds. In this study, GCMS method is central for identification of these phytoconstituents. This method is a direct and fast analytical approach for identification of terpenoids and steroids requiring only a few grams of plant material. And also this method can be applied to the analysis of any other low concentration minor component fatty acids in complex sample matrices.

ACKNOWLEDGMENTS

We are grateful to the Department of Pharmaceutical Laboratory GAU- Jamnagar for providing work

Table 1. Fatty acid methyl ester identified in *C. dichotoma* bark

Peak#	RT	Name of the components	Molecular formula	MW	Peak area %
1	2.11	Pentene 2,2 di methyl	C_7H_{16}	100	1.18
2	2.35	2-methyl pentane	C_6H_{14}	86	18.29
3	2.50	Sec-butylcarbinol	$C_5H_{12}O$	88	16
4	2.65	Bis-isopropyl	C_6H_{14}	86	20.79
5	2.91	Undecane	$C_{11}H_{24}$	156	4.12
6	2.96	3-methyl heptane	C_8H_{18}	114	3.14
7	3.07	1-nonene, 2-methyl	$C_{10}H_{20}$	140	18.34
8	3.35	Di isoamyl	$C_{10}H_{22}$	142	0.28
9	3.43	2,4–dimethyl-3-ethyl pentane	C_9H_{20}	128	0.97
10	3.54	Cicloesano	C_6H_{12}	84	12.47
11	3.65	Sextone	C_7H_{14}	98	0.3
12	3.77	Gem-di methyl cyclopentane	C_7H_{14}	98	0.31
13	3.82	1,2,3, tri methyl cyclopentane	C_8H_{16}	112	0.21
14	3.89	Butyric acid-2,2 di methyl vinyl ester	$C_8H_{14}O_2$	142	0.93
15	15.82	Hexadecanoic acid, methyl ester or palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.40
16	20.57	Heptadecanoic acid methyl ester or margaric acid methyl ester	C18H36O2	284	0.52
17	22.42	9-octadeconoic acid(Z)-, methyl ester or oleic acid, methyl ester	C19H36O2	296	1.09

place to undertake this study and also thankful to the Department of Chemistry, Saurashtra University for providing instrumental facilities.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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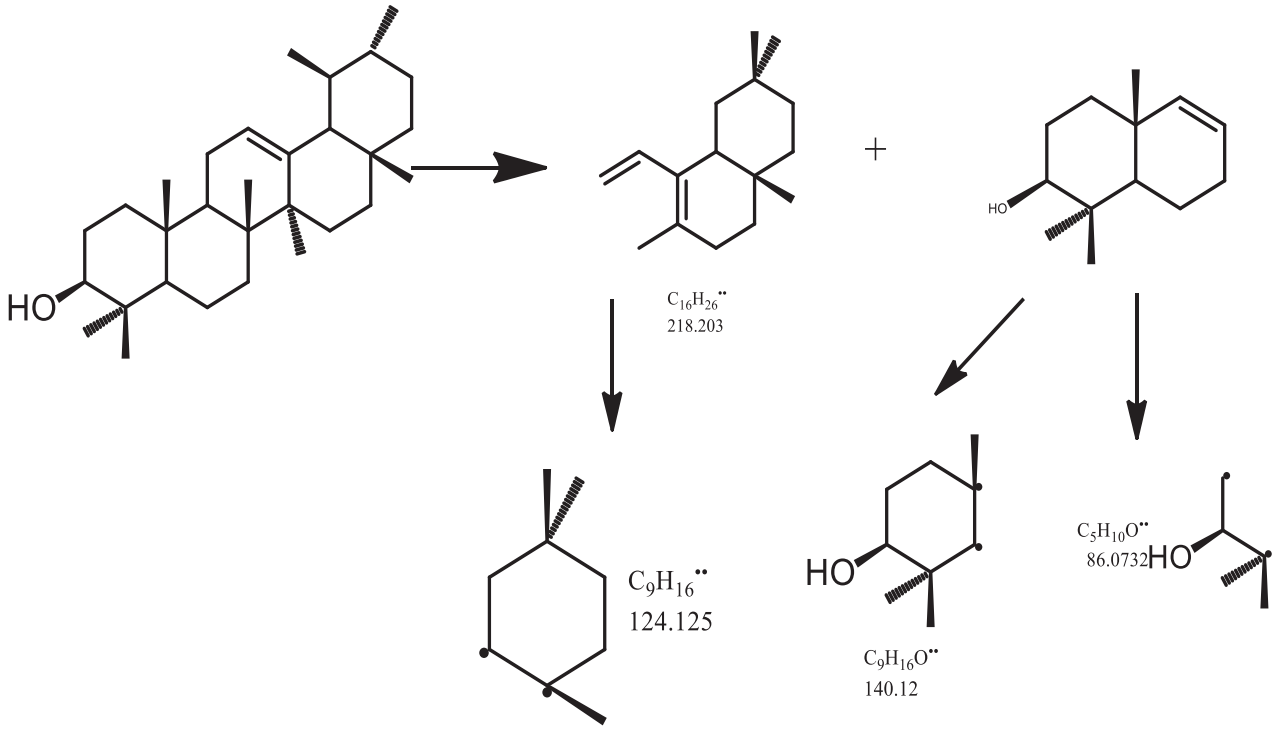

Fragments

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Содержание тритерпеноидов и жирных кислот в коре Cordia dichotoma (Forst f.)

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Цель: Изолировать и определять ингредиенты коры Cordia dichotoma (Forst f.) - растения, используемого в лечебных целях в народной медицине.

Материалы и методы: В этом исследовании использовали экстракт петролейного эфира. Было проведено омыление для удаления жирных кислот из неомыляемых веществ.

Результаты: Тритерпеноид-α-амирин был выделен из коры изократическим моющим средством. Новоизолированные химические соединения были анализированы с помощью GC/MS, инфракрасного излучения (ИК) и ультрафиолетового излучения (УФ). Химический состав метиловых эфиров жирных кислот FAMEs в коре Cordia dichotoma также анализировали с помощью масс-спектрометрической газовой хроматографии. После метиловой этерификации было установлено наличие 17 компонентов в коре. Для валидации этого метода были исследованы условия дериватизации.

Вывод: Настоящий анализ показал, что кора Cordia dichotoma содержит 17 жирных кислот. Основные положения этой статьи посвящены разработке и применению хроматографических методов разделения, изолирования и идентификации компонентов.