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Identification and structural elucidation of linolenic acid in the ethanolic fruit extract of *Cucumis Trigonus*

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ABSTRACT

The ethanolic fruit extract of *Cucumis trigonus* Roxb. of family Curcurbitaceae was subjected to isolation and identification of chemical constituents. The extract was purified and isolated by column chromatography and thin layer chromatography (TLC). The isolated compound was then subjected to Infra red spectroscopy (IR) for identification of functional groups, ^{1}H NMR and ^{13}C NMR for identification of protons and carbon atoms. LCMS and elemental analysis were done for identifying the molecular weight and elemental composition of the isolated compound. From the spectra obtained from IR, ^{1}H NMR and ^{13}C NMR, LCMS and elemental analysis, the isolated compound was found to be linolenic acid ($C_{18}H_{30}O_2$) with a molecular weight of 278.43.

Introduction

Plants have the ability to synthesize secondary metabolites to defend them against their predators. Some compounds turn out to have beneficial effects towards human diseases [1]. Secondary metabolites are highly varied in structure, many are phenolic aromatic substances or oxygen substituted derivatives [2]. Many of the herbs and spices used by human yield useful medicinal compounds [3].

Many conventional drugs originate from plant sources and most of the few effective drugs were plant based. Examples include aspirin from willow bark, digoxin from foxglove, quinine from cinchona bark, and morphine from the opium poppy. The development of drugs from plants continues with drug companies engaged in large scale pharmacological screening of herbs [4]. Indian Ayurveda medicine has been using herbs such as turmeric as early as 1900 B.C. [5]. The *Sushruta Samhita* in the 6th century BC describes 700 medicinal plants, 64 preparations from mineral sources, and 57 preparations based on animal sources [6].

Numerous molecules have come out of Ayurvedic experiential base, examples include rauwolfia alkaloids for hypertension, holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic agents, mucuna pruriens for Parkinson's disease, piperidines as bioavailability enhancers, baccosides in mental retention, picrosides in hepatic protection, phyllanthins as antivirals, curcumine in inflammation, many other steroidal lactones and glycosides as immunomodulators. A whole range of difficult to treat diseases such as cancers, cardiovascular diseases, diabetes, rheumatism and AIDS is the requirement of new effective drugs. Most developing countries have relied and will continue to rely on traditional natural medicines due to the high costs of modern allopathic medicines [7].

Cucumis trigonus Roxburghii of family Curcurbitaceae is a perennial scabrid monoecious tendrillar herb with slender angled stem, leaves deep palmately five lobed, hispid on the nerves beneath and rounded at the apex. Male flowers are small and are found in clusters where as female flowers are solitary. Fruits are ellipsoid or sub-global, yellow or yellow with green stripes, seeds are white and ellipsoid [8]. The plant is distributed throughout India and found in areas of Ceylon, Afghanistan, Persia and Northern Australia [9]. Roots, fruits and seeds are the extensively used medicinal parts of the plant. Roots are purgative and liver tonic, fruits are used for stomachic, ascites, anemia and constipation and acts as a diuretic. Seeds have unsaturated lipids as major constituents and acts as a coolant and astringent.

The present study deals with the extraction and characterization of ethanolic fruit extract of *Cucumis trigonus*. The characterization of the extract includes the isolation and purification using the column and thin layer chromatography. The isolated compounds from TLC were subjected to various instrumental analysis like IR, ^{1}H NMR and ^{13}C NMR were done to identify the presence of functional groups, protons and carbon atoms respectively. LCMS and elemental analysis were done to identify the molecular weight and the elemental composition of the isolated compound.

Materials and methods

Extraction

350g of the dried fruits of *Cucumis trigonus* was soaked in 1L acetone and allowed to stand for 24 hr. filtered the solvent and extraction was repeated thrice by adding 1L of acetone each time. Collected all the three extracts and concentrated using rotary vacuum to get 20g of crude extract.

Isolation, purification and identification

Column chromatography

20g of the crude extract was re dissolved in minimum quantity of acetone. To this 60g of silica gel (60:120 Mesh) was added and allowed to dry to get free flow of admixture. This admixture was packed for column chromatography and column was eluted using increasing polarity of solvent as mentioned in table 1.

Fraction 55th- 68th were combined and further treated with charcoal to get the pure compound.

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The compounds obtained from column chromatography were separated and concentrated. The compounds were then tested using TLC for identification.

Thin layer chromatography (TLC)

Coated the slurry (1:2) over the glass plates at a thickness of 0.25mm and allowed to dry at room temperature for 15-30 min. heated the plates in an oven at 100-120°C for 1-2 hr. to remove the moisture and to activate the adsorbent on the plate. The column eluted was applied at 2.5cm from one end of the glass plate. Allow the samples to dry so that spotting can be done repeatedly for a more concentrated samples spot. Pout the developing solvent into tank to a depth of 1.5cm. After equilibration, remove the cover plate and place the thin layer plate (sample applied) vertically in the tank so that it stands in the solvent with the spotted end dipping in the solvent. The separation of the compounds occurs as the solvent moves upward. Once the solvent reaches the top of the plate remove it from the tank, dry and spray with the spraying reagent for the identification of the separated compounds. The samples produce colour with the spraying agent.

Infra red (IR) spectral studies

The infrared spectrum originates from the vibrational motion of the molecule. The vibrational frequencies are a kind of fingerprint of the compounds. This property is used for characterization of organic, inorganic and biological compounds. The band intensities are proportional to the concentration of the compound and hence qualitative estimations are possible. The IR spectroscopy is also carried out by using Fourier transform (FT) technique.

Description

The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise o the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer.

The Shimadzu Spectrum1 FT-IR instrument consists of globar and mercury vapour lamp as sources, an interferometer chamber comprising of KBr and mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm^{-1} is covered by this instrument. The spectrometer works under purged conditions. Solid samples are dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a typical resolution of 1.0 cm^{-1}. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible.

^{1}H NMR and ^{13}C NMR spectral studies

FT-NMR spectroscopy is used to determine the molecular structure based on the chemical environment of the magnetic nuclei like ^{1}H, ^{13}C, ^{31}P, etc., even at low concentrations. This is one of the most powerful nondestructive techniques in elucidating the molecular structure of biological and chemical compounds.

Description

In FT NMR spectroscopy, a strong RF pulse excites the entire range of processional frequencies of a given nuclear species whose time response is known as free induction decay (FID) containing all the information. A Fourier transform of FID gives the NMR spectrum. This is a much faster technique compared to continuous wave and hence it is possible to detect weak lines by signal averaging methods. This technique is used in JEOL, GSX 400 NB FT-NMR spectrometer. The spectra of samples containing low abundant nuclei like ^{13}C, ^{31}P, etc., are thus easily obtained. Also dynamic studies are possible by relaxation measurements. Homo and hetero ^{1}H decoupling are also possible.

JEOL GSX 400 NMR operates at 400 MHz (for proton) with a magnetic field of 9.3 Tesla. Hence a supercon magnet is used. A PDP-11/73 computer is an integral part of the instrument for the purpose of Fourier transformation and spectral manipulation. The spectrum is plotted on a HP plotter and data can be obtained on a printer. The probes available are ^{1}H/^{13}C combined and multinuclei probe to study the nuclei like ^{23}Na, ^{27}Al, ^{43}Ca, ^{37}Cl, ^{79}Br, etc., except ^{19}F, ^{3}H and T_1. Among other things, many types of 2D spectral measurements are possible. It is possible to obtain high resolution NMR spectra of many compounds using the solids accessory employing MAS technique which otherwise are insoluble in usual solvents. Though the resolution is poor, this is also useful for polymers, etc.

Sample required

5 mg for ^{1}H and 15mg for ^{13}C and other nuclei. Solubility: 10mg / ml for ^{1}H and 50 mg / ml for ^{13}C and others. Solvent must be specified and solubility must be checked before injecting the samples in order to save the expensive deuterated solvents. Sample must be free of paramagnetic impurities. Solvents available: $CDCl_3$, D_2O, C_6H_6, CD_3COCD_3 and $DMSOd_6$.

Determination of molecular weight of the compound by liquid chromatography mass spectroscopy (LC MS)

Mass spectrometry has become a vital tool in the hands of organic chemists and biochemists because of its potential to supply definitive, qualitative and quantitative information on molecules based on their structural compositions.

Description

The mass spectrometer consists of an ion source, an analyzer and a detector maintained at a vacuum of 10^{-8} torr. The vaporized molecules are first bombarded by a stream of high energy electrons converting some of the molecules into molecular ions and fragment ions. The ions are accelerated and separated according to their mass to charge ratios in the magnetic field (analyzer). These are then velocity focused in an electric field. The ions are detected in terms of their mass to charge ratios by the detector namely a secondary electron multiplier. The output is amplified and fed to the recorder for processing. The mass spectrum, a graph of intensity of the ions detected vs. m/z value is presented on the screen and printed. An IBM compatible PC is used to control the Mass spectrometer and also to acquire process and print out the spectral data.

The 410 Shimadzu Binary LC 500 Mass spectrometer with data system is a high resolution, double focusing instrument with reverse Nier-Johnson geometry. The maximum resolution is 48000 at 10% valley in low resolution mode. Maximum calibrated mass is 2000 Daltons.

Sample required

About 1 mg of the sample required can be in the solid or liquid state. Sample should be pure and free from solvents and metal ions.

Identification of elements by elemental analysis

The carbon, hydrogen, nitrogen and oxygen contents of the compounds were analyzed by Erlinmeier flask method.

Structural elucidation

The spectra of IR, ^{1}H NMR and ^{13}C NMR, elemental analysis and LC MS were used for structural elucidation of the isolated compounds.

Results

The pure compounds were subjected to various instrumentation analyses like IR, ^{1}H NMR, ^{13}C NMR, LCMS and elemental analysis for structural elucidation. IR spectra of the isolated compounds were analyzed using Shimadzu FT-IR spectrophotometer 500-4000 cm^{-1}. The IR spectrum of the selected compound was recorded. ^{1}H NMR and ^{13}C NMR spectra of the isolated compounds were done using Bruker 400 NB, 400 MHz FT NMR spectrometer. The ^{1}H NMR and ^{13}C NMR spectrum of the selected compounds were recorded. LCMS was done by Shimadzu MAT 8230 JEOL LC mate LCMS spectrometer. The mass spectra of the selected compounds were recorded. The carbon, hydrogen and oxygen contents of the purified compound were analyzed by Erlenmeyer flask method. The graphical representation of the compounds was recorded.

Compound number: 1

Compound code : BRK-2

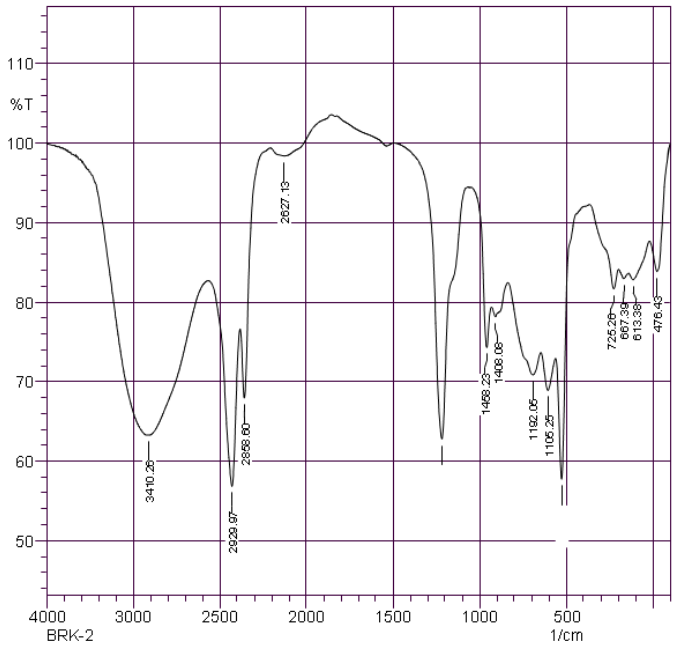
Colour of the compound: Clear yellowish liquid

Melting Point: -11°C

Identification of functional groups by IR spectral studies

IR spectrum of the isolated compound registered a broad band at 3410.26 cm^{-1} and a stretching band at 1716.70 cm$^{-1,}$ this indicates the presence of OH group and a carbonyl (C=O) group. The spectrum also shows peaks at 2929.97 cm^{-1}, 1458.23 cm^{-1} and 1408.08 cm^{-1} which indicates the presence of aliphatic stretching. The spectrum is depicted in the figure 1.

Fig. 1: IR spectrum of the isolated compound 1



Structural elucidation of active constituent by ^{1}H NMR and ^{13}C NMR spectral studies

^{1}H NMR spectrum of the isolated compound BRK-2 registered singlets at δ 0.92 for methyl group and δ 3.20 for hydroxyl group. The spectrum shows multiplets at δ 1.18 – 1.42 for 20 aliphatic protons, δ 4.3-5.1 for C_9, C_{10}, C_{12} and C_{13} protons and δ 5.5-6.0 for C_{15} and C_{16} two olefinic protons.

^{13}C NMR spectrum showed a peak at δ 170 for the presence of carbonyl (C=O) carbon and peaks at δ141.40, δ 140.46, δ 135.78, δ 134.43, δ134.73 and δ 131.35 for six olefin carbons and other carbon peaks at δ 49.40, δ 45.38, δ 45.36, δ 44.65, δ 41.38, δ 31.33, δ 30.83, δ 29.46, δ 26.28, δ 24.33, δ 21.27 and δ 18.36 for aliphatic carbons from C_2 to C_8, C_{11}, C_{14}, C_{17} and C_{18} respectively. The ^{1}H NMR and ^{13}C NMR spectra are given in the figure 2 and 3 respectively.

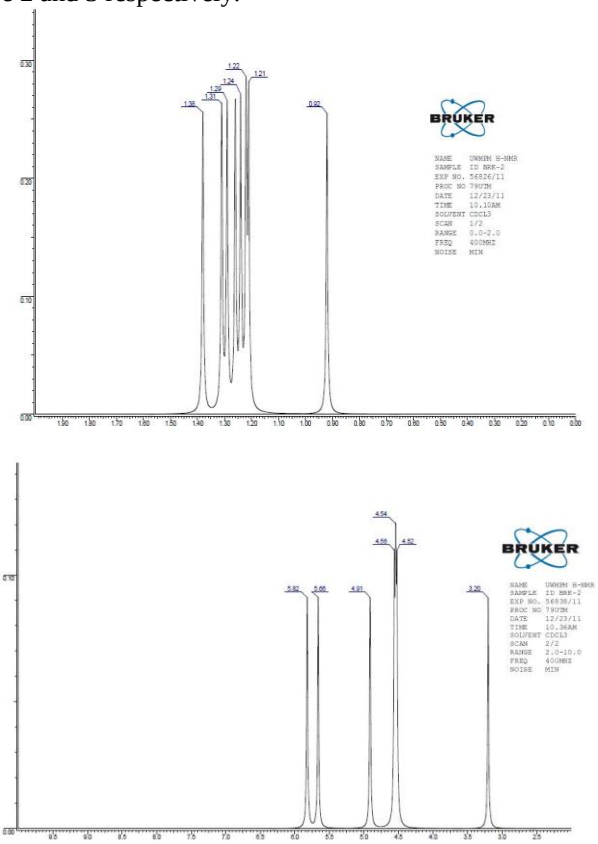


Fig.2. ^{1}H NMR spectra of the isolated compound 1

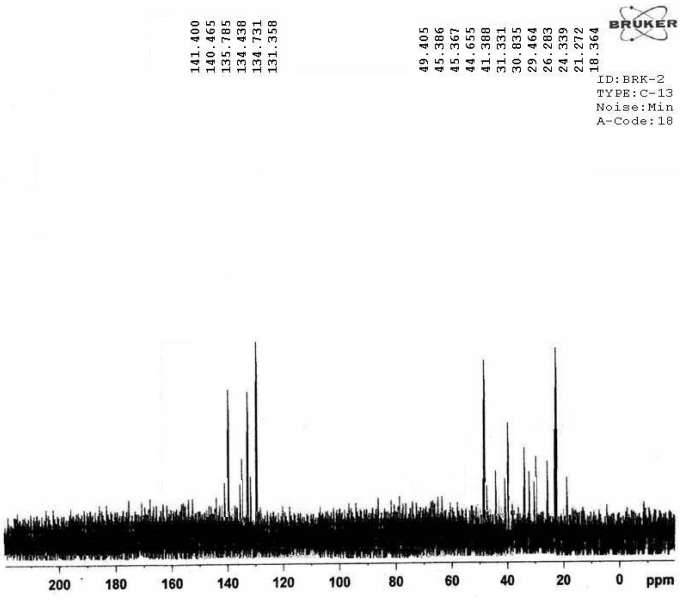


Fig. 3. ^{13}C NMR Spectrum of the isolated compound 1

Identification of active constituent by mass spectrum

Mass spectrum showed molecular ion peak at m / z 278 and (m+1) peak at 279 and other fragment ion peaks at 263, 179 and 146. The mass spectrum of the compound BRK-2 is shown in figure 4.

Identification of active constituent by elemental analysis

The elemental composition of the isolated compound by elemental analysis is given in the figure 5. The elemental analysis showed the presence of 77.65% of carbon, 10.86% hydrogen and 11.49% of oxygen.

From the mass spectrum and the elemental analysis of the isolated compound, the molecular weight was confirmed as 278.43 and molecular formula was $C_{18}H_{30}O_2$ and the structure elucidated is given in figure 6.

All our spectral data coincide with the spectral data of linolenic acid, which confirms that the isolated compound is linolenic acid.

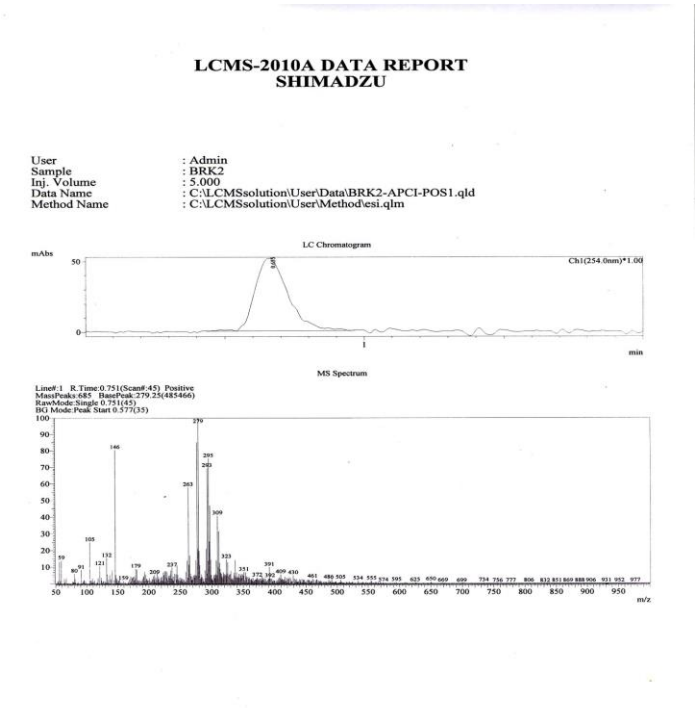


Fig.4. Mass spectrum of the isolated compound 1

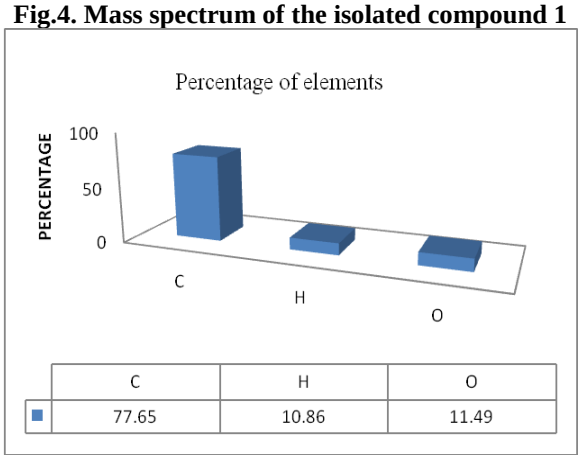


Fig.5. Elemental analysis of isolated compound 1

Molecular formula: $C_{18}H_{30}O_2$

Molecular weight: 278.43

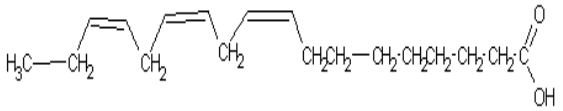


Fig.6. Structure of the isolated compound 1

Compound name: (9*Z*, 12*Z*, 15*Z*)-octadeca-9, 12, 15-trienoic acid; alpha linolenic acid

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Table1. Column chromatographic solvent systems for the separation of active constituents

S.No	% of solvents	Fraction number	Vol. of solvent
1	100%Hexane	1-6	300
2	5%E.Ac/hexane	7-16	500
3	10%E.Ac/hexane	17-24	400
4	15%E.Ac/hexane	25-34	500
5	20%E.Ac/hexane	35-44	500
6	25%E.Ac/hexane	45-50	300
7	30%E.Ac/hexane	51-58	400
8	35%E.Ac/hexane	59-64	300
9	40%E.Ac/hexane	65-70	300

E.Ac= Ethyl acetate

Fraction 55th- 68th were combined and further treated with charcoal to get the pure compound.