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Navya AS
Department of Pharmacognosy, Government College of Pharmacy, Bengaluru, Karnataka, India

Anitha S
Department of Pharmacognosy, Government College of Pharmacy, Bengaluru, Karnataka, India

Correspondence
Navya AS
Department of Pharmacognosy, Government College of Pharmacy, Bengaluru, Karnataka, India

Isolation of compound (CA-01) from the bark of *Careya arborea* by using column chromatography

Navya AS and Anitha S

Abstract
Careya arborea Roxb, commonly known as wild guava. Belonging to the family Lecythidaceae is known as Padmaka in Ayurveda. It is used as astringent, demulcent, antitumour, antipyretic and antipruritic, hepatoprotective, antimicrobial, antioxidant, CNS activity, antileishmanial, anticoagulant, analgesic, cytotoxic activity. The authenticated barks were procured from Sri Venkateshwara University, Tirupathi. The coarse powder of bark of *Careya arborea* subjected for extraction process by using soxhlet apparatus by using successive solvents. The methanol solvent produces highest extractive yield. Therefore the methanolic extract of bark was used for optimization of solvent systems for TLC studies. The mobile phase selected was n-butanol: glacial acetic acid: water (4:1:5) showed best pattern of band separation. So that the methanolic extract was subjected to column chromatography. Therefore the extract packed into column and eluted with hexane: acetone to obtain 11 fractions. The 10th fraction showed the three bands with similar chromatographic pattern by TLC identity tests. This fraction was subjected for preparative column for isolation and purification of compound. Purity of the isolated compound was determined by HPLC analysis. Structural determination/ characterization was done by UV, IR spectroscopic analysis.

Keywords: *Careya arborea*, HPLC, isolation, characterization, chromatography

1. Introduction

Isolation of active constituents: The investigation of the bioactive natural products has assumed a greater sense of urgency in response to the expanding human population and its subsequent demand for food, good health and increasing areas of land on which to live. Nature recognizes no artificial barrier such as those of the academic disciplines and thus it is no surprise to find investigators with quite different academic training studying various aspects of bioactive natural products.

As the herbal drugs contains so many chemical compounds, It is essential to separate out those compounds which are responsible for therapeutic effect which are called as active constituents. The use of pure active constituents is obvious since the compound is having a fixed and definite physiological effect without the presence of such an extraneous factor like other active constituents still unknown. Isolation is a part of natural product chemistry, through which it is possible to separate different components and biologically active ones can be incorporated as ingredients in the modern system of medicine. The column chromatography technique (Adsorption chromatography) is widely used for the separation, isolation and purification of chemical compounds from natural drugs.

Extraction and Isolation: Extraction from the specific plant material is a trial and error exercise, in which different solvents are tried under variety of conditions such as time and temperature of extraction. The success or failure of the extraction process is monitored by the most appropriate assay.

Once extract from the plant, the bioactive component then has to be separated from the co-extractive. This may involve simple crystallization of the compound from the crude extract or more usually it may involve further solvent partition of the co-extractive and extensive chromatography, taking advantage of particular properties of the desired compound, such as acidity, polarity and molecular size.

Final purification, to provide compounds of suitable purity for the structural analysis, may be accomplished by appropriate techniques such as recrystallization, sublimation or distillation.

Structure determination: The process of structural determination involves accumulating data from numerous sources, each of which gives some structural information, and the

assimilation of these data into a chemical structure that uniquely fits all the available structural information. A wide range of spectroscopic instrumentations such as UV, IR, NMR, and Mass Spectroscopy [1].

The bark of *Careya arborea*: The bark of *Careya arborea* contains chemical constituents such as terpenoids, flavonoids, coumarins, saponins, tannins etc.

Ethnopharmacological action of bark of *Careya arborea* – As an Astringent, demulcent, emollient embrocations, snake bite, antipyretic and antipruritic, anthelmintic, antidiarrhoeal, epileptic fits, treatment of tumour,bronchitis [2].

2. Materials and Methods:

2.1 Collection and authentication of plant material

The dried bark of *Careya arborea* was collected from Kerala. Identification and authentication of plant material was done by Dr. K Madhava Chetty, Asst. Professor, Dept. of Botany, Sri Venkateshwara University, Tirupathi. And the drug was subjected for physicochemical and pharmacognostic evaluation.

2.2 Preparation of extracts

The coarse powder of bark of *Careya arborea* subjected for extraction process by using soxhlet apparatus by using successive solvents. The methanol solvent produces highest extractive yield. Therefore extraction process was continued by using methanol solvent. 50gms of coarsely powdered drug of bark was packed into a thimble each time and extracted with solvent methanol. After the effective extraction, the solvent was distilled off, the extract was then concentrated on water bath and the extract obtained was weighed. The extractive yield was calculated on air dried basis. The extract were concentrated by evaporating the solvent and stored at 4 ± 1.0º C [3].

2.3 Optimization of TLC system for methanolic extract

Various solvent systems were developed for TLC identification of constituents in the extract and the one showing efficient and clear separation was selected as mobile phase for the study.

Activation of the plates: The pre-coated plates were placed in a hot air oven and heated @ 105 °C for 2hrs [4 and 5].

Stationary phase: Precoated Silica gel 60 F_{254} (0.25µ) plates (Merk)

Mobile phase: n-butanol: Acetic acid: Water, Benzene: Metanol: Acetic acid, Hexane :Ethyl acetate, n-Butanol: Water, Pet ether : Ethylacetate.

The sample was prepared using 2mg of methanolic extract was dissolved in 5ml of methanol and it was visualized in UV at 254nm.

Method: Sample solutions were applied in the form of bands on activated TLC plates using capillary tubes and developed in TLC chamber using suitable solvent system. Plates were air dried and observed under UV at 254nm. The solvent systems tried for the optimization of TLC solvent system are given in table 1.

Table 1: List of various solvent systems used for TLC optimization of *Careya arborea* extracts

Sl. No.	Solvent system	Composition
1.	Hexane : Ethyl acetate	1:1
2.	Benzene : Methanol : Acetic acid	45:8:4
3.	n-butanol : Glacial acetic acid : Water	4:1:5
4.	n-Butanol : Water	1:1
5.	Pet ether : Ethyl acetate	1:1

2.4 Separation of phytoconstituents using column chromatography: The extract was subjected for separation of phytoconstituents by using column chromatography.

2.5 Pre-column preparation: The pre-column preparation includes adsorption of the selected extract, charging and saturation of the column.

a. **Adsorbent:** silica gel of various particle size, alumina, Diaon, amberlite, deolite, sephadex etc.
b. **Preparation of slurry:** Combine the solid stationary phase with a small amount of nonpolar solvent in a beaker. Thoroughly mix the two until a consistent paste is formed, but is still capable of flowing.
c. **Column specification:** A glass column of 600mm length and 50mm width having 750ml capacity was used.
d. **Preparation of the extract:** The methanolic extract was dissolved in methanol. Then it is adsorbed with silica gel in a ratio of 1:2 (drug: silica gel) and dried at 60ºC, under 600 mm/Hg in VTD.
e. **Packing of column:** A glass column was rinsed with acetone. A cotton disc was placed at the bottom then homogeneously mixed slurry was poured into the column as carefully as possible. The slurry method normally gives the best column packing. The most important aspect of packing the column is creating an evenly distributed and packed stationary phase because cracks, air bubbles and channeling will lead to a poor separation. The column was charged with extract and cotton was placed over the charged material for further elution with mobile phase.
f. **Selection of mobile phase:** The solvent system was selected from the TLC optimization studies.
g. **Collection of fractions:** The various fractions were collected by changing the proportion of mobile phase. All the fractions were concentrated and subjected for TLC studies.

2.6 Column chromatography of methanolic extract using silica gel column (1):

5 g of methanolic extract was mixed with 10 gm of silica gel(#60-120) ethanol is used as a solvent in the ratio 1:2 (drug to silica gel) and air dried at room temperature. A column of 500 ml capacity. 15 g of silica gel mixed material was charged and the elution was started with Pet. ether and then followed by solvent such as hexane and acetone [6 and 7]. The fractions collected were concentrated using flash evaporator (Table 3). The specifications of the column chromatography are given below (Table 2).

Requirements

- Stationary phase: Silica gel (60-120# mesh).
- Mobile phase: Hexane : acetone
- Charged material: Methanolic extract

Table 2. Column chromatography specification

Adsorbent used	Silica gel (60-120 mesh)
Amount of adsorbent Packed	100gms
Charged material	Methanolic extract (15gms)
Column Specifications: Length(cm) Diameter(mm)	 600mm 50mm
Amount of Extract used	5gms
Extract : Adsorbent ratio	1:2
Volume of each fraction collected	100ml

Table 3. Fractions collected from Column Chromatography

Fraction Code	Solvent system	Ratio	Volume collected (ml)
C1-Fr1	Hexane	100%	100
C1-Fr2	Hexane : Acetone	90:10	100
C1-Fr3	Hexane : Acetone	80:20	100
C1-Fr4	Hexane : Acetone	70:30	100
C1-Fr5	Hexane : Acetone	60:40	100
C1-Fr6	Hexane : Acetone	50:50	100
C1-Fr7	Hexane : Acetone	40:60	100
C1-Fr8	Hexane : Acetone	30:70	100
C1-Fr9	Hexane : Acetone	20:80	100
C1-Fr10	Hexane : Acetone	10:90	100
C1-Fr11	Acetone	100%	100

2.7 Thin layer chromatographic study of fractions collected from column

Stationary phase and mobile phase used was silica gel 60 F_{254}.and n-Butanol: Glacial acetic acid: Water (4:1:5) respectively. The sample was prepared using 1mg of fractions were dissolved in 2ml of methanol and it was visualized in UV 254nm.

Procedure for development of TLC plate

The sample solutions were applied in the form of bands on activated TLC plates using capillary tube and developed in TLC chamber using mobile phase. Plates were dried and observed under UV at 254nm.

2.8 Isolation of compound 1 (CA-01)

The 10th Fraction of column 1 was collected, dried and used for TLC identity test.

Thin Layer Chromatographic study of C1-F10 fraction.

TLC studies showed a single band in UV at 254nm in the solvent system n-Butanol: Glacial acetic acid: Water (4:1:5). The compound was coded as CA–01. The compound was subjected to HPLC.

HPLC of the isolated compound CA-01

The compound was subjected for analytical HPLC for assessing its % purity with necessary modification in HPLC method. Methanol: Water was used to assess the percentage purity of the compound. Specified quantity of the isolated compound was dissolved in methanol. 20µl of the sample was injected into the column and chromatogram was recorded (Table 4).

Table 4. HPLC requirements for analysis of isolated compound.

Instrument	SHIMADZU HPLC system LC 2010HT with UV detector in combination with Class LC solution software		
Column	LiChrospher C-18 (250 X 4.6 X 5µ)		
Flow rate	1.0 ml/min		
Mobile phase	Pump A	Methanol	
	Pump B	Water	
Isocratic elution	Time	B. concn.	A. concn.
	15	10%	90%
Injection volume	20µl		
Detector	UV detector.		

HPLC of the purified compound CA-01.
The compound was subjected for analytical HPLC for assessing its % purity. The HPLC requirements are mentioned in Table 5.

Sample preparation: 2mg of CA-01 was dissolved in methanol with the help of sonication. It is then filtered using HPLC filter and used.

Procedure: The HPLC of the CA-01 was performed using above-mentioned protocol and the chromatogram was recorded**.**

Table 5. HPLC requirements for analysis of isolated compound.

Instrument	SHIMADZU HPLC system LC 2010HT with UV detector in combination with Class LC solution software		
Column	LiChrospher C-18 (250 X 4.6 X 5µ)		
Flow rate	1.0 ml/min		
Mobile phase	Pump A	Methanol	
	Pump B	Water	
Isocratic elution	Time	B. concn.	A. concn.
	15	10%	90%
Injection volume	20µl		
Detector	UV detector.		

2.9 Compound Profile
Physical properties of isolated compound: The isolated compound was analyzed for its physical properties like appearance, colour, texture and nature.

Physicochemical properties of isolated compound: Physicochemical properties like solubility, melting point was determined for isolated compound using standard procedure.

Spectral analysis of isolated compound CA-01: The isolated compound was subjected for spectral studies for characterization.

UV spectroscopy: UV absorption of isolated compound was carried out in the UV range and the absorption maxima were found to be 272 nm.

IR Spectral studies: FT-IR was carried in the mid IR range to find out the functional group present in the isolated compound. IR spectra of the isolated compound showed the peaks in the range between 3400-600cm^{-1} [8 and 9].

3. Results and Discussion
The coarsely powdered drug was initially. Extracted with successive solvents, further its showed highest percentage extraction with methanol solvent (4.86%) (Table 6).

Table 6. Percentage yield of extracts obtained by successive solvent extraction

Sl.no	Parts of the plant	Solvents	Weight of drug taken (g)	Weight of extract (g)	Yield (%)
1	Barks	Pet.ether	50	0.19	0.38
		Chloroform	50	0.47	0.94
		Methnol	50	2.43	4.86
		Ethanol	50	1.85	3.70
		Water	50	1.02	2.04

To optimise the for the solvent system for the active methanolic extract different solvent systems was used (Table 7). Out of various solvent systems n-butanol: Glacial acetic acid: water was found to be the most suitable solvent system with a maximum Rf value (0.7,0.82 and 0.74)and its TLC profile shown maximum number of bands (Fig 1).

Table 7. Optimization of solvent system for active methanolic extract

Sl. No.	Solvent system	Detection using UV at 254 nm	
		No. of bands	Rf value
1.	Hexane : Ethyl acetate	Single band	0.6
2.	Benzene : Methanol : Acetic acid (45:8:4)	No separation	-
3.	n-butanol : Glacial acetic acid : Water(4:1:5)	Three bands	0.7,0.82,0.74
4.	n-butanol : Water (1:1)	Single band	0.32
5.	Pet ether : Ethyl acetate (1:1)	No separation	_

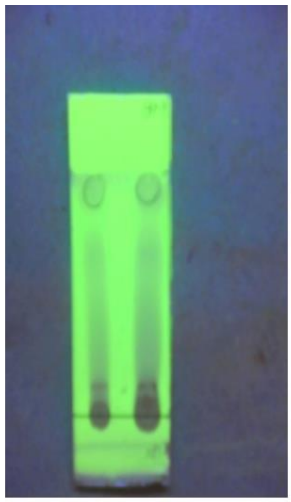


Fig 1. Sample visualization by UV at 254 nm

Column chromatography of methanolic extract: Methanolic extract was subjected for separation of constituents using Silica gel column chromatography using diiferent solvents with 11 fractions. The Cl-Fr10 initially indicates the presence of compound with pale yellow colour (Table 8).

Table 8. Details of fractions collected from column chromatography

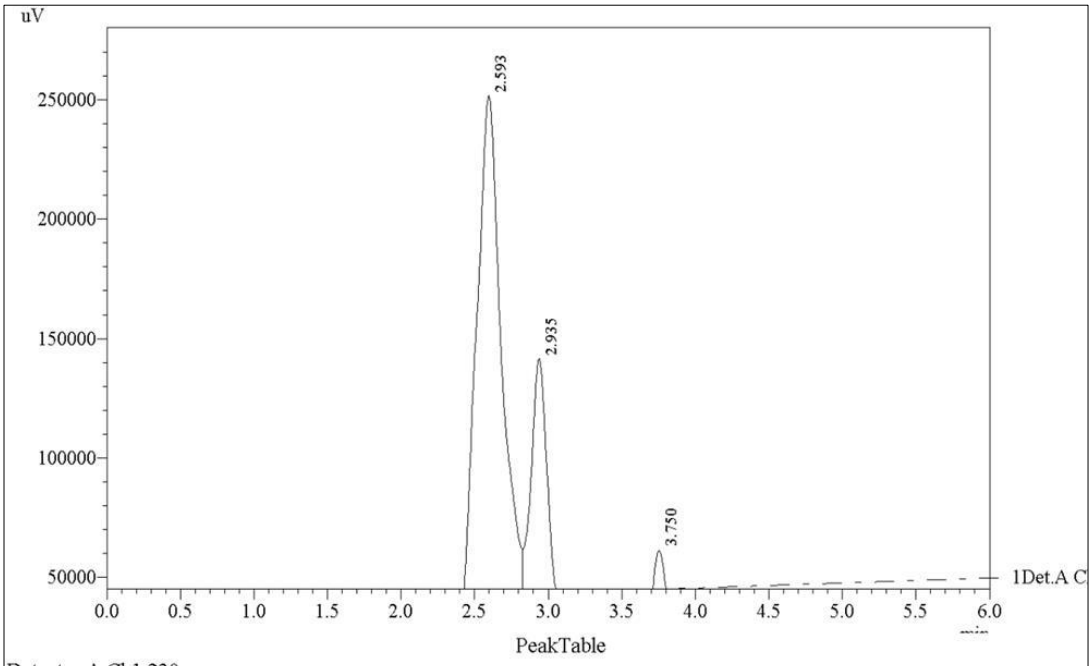
Fraction Code	Solvent system	Ratio	Colour
C1-Fr1	Hexane	100%	Colourless
C1-Fr2	Hexane:Acetone	90:10	Colourless
C1-Fr3	Hexane:Acetone	80:20	Colourless
C1-Fr4	Hexane:Acetone	70:30	Colourless
C1-Fr5	Hexane:Acetone	60:40	Colourless
C1-Fr6	Hexane:Acetone	50:50	Colourless
C1-Fr7	Hexane:Acetone	40:60	Colourless
C1-Fr8	Hexane:Acetone	30:70	Colourless
C1-Fr9	Hexane:Acetone	20:80	Colourless
C1-Fr10	Hexane:Acetone	10:90	Pale yellow
C1-Fr11	Acetone	100%	Colourless

TLC profile study: The TLC profile study of eleven fractions collected from column (1) revealed that Cl-Fr10 initially showed there bands with Rf value of 0.5, 0.58 and 0.6. It indicates the presence of compound (Table 9).

Table 9. TLC study of fractions collected from the column

Fraction Code	No of bands	Rf value
C1-Fr1	0	0
C1-Fr2	0	0
C1-Fr3	0	0
C1-Fr4	0	0
C1-Fr5	0	0
C1-Fr6	0	0
C1-Fr7	0	0
C1-Fr8	0	0
C1-Fr9	0	0
C1-Fr10	Three bands	0.5,0.58,0.6
C1-Fr11	0	0

HPLC study: The HPLC profile of 10th fraction collected from column (1) was subjected for HPLC study. Based on the HPLC chromatogram it reveals that the presence of three compounds with maximum peak and retention time of 2.593, 2.935 and 3.750 (Fig 2).

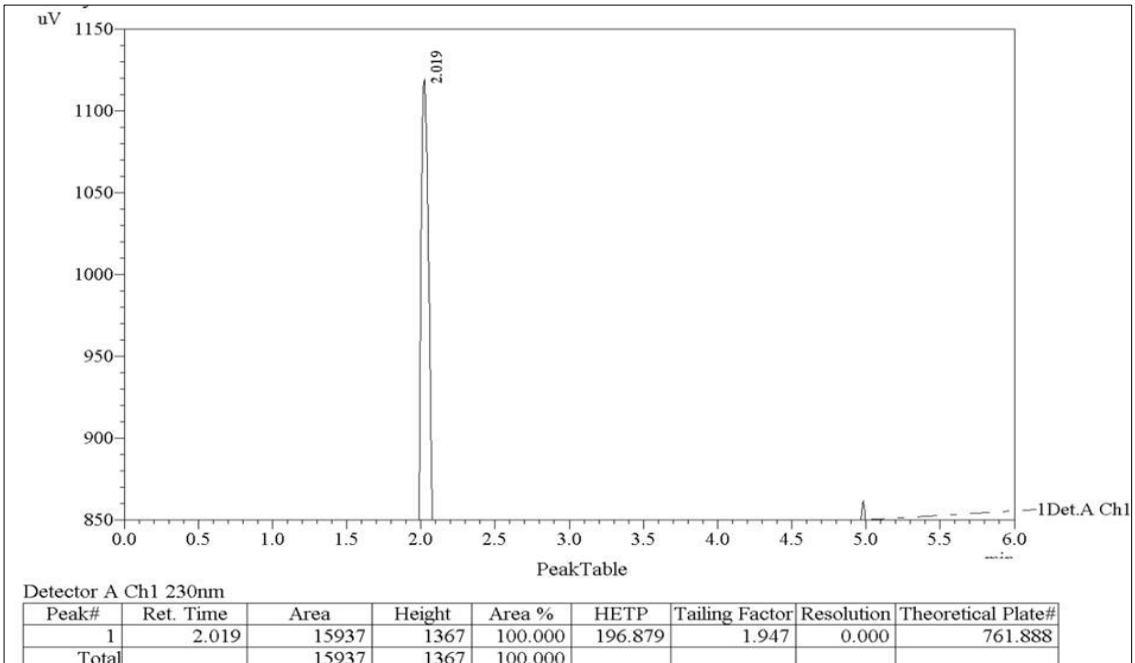


Detector A Ch1 230nm

Peak#	Ret. Time	Area	Height	Area %	HETP	Tailing Factor	Resolution	Theoretical Plate#
1	2.593	3730516	257622	58.967	199.696	0.000	0.000	751.141
2	2.935	1909963	147213	30.190	89.311	0.000	1.028	1679.522
3	3.750	685928	65332	10.842	45.593	0.000	2.973	3289.977
Total		6326407	470167	100.000				

Fig 2. HPLC chromatogram of fraction 10

The HPLC profile of 10th fraction collected from column (1) was further subjected for HPLC study for accessing the percentage purity. The HPLC chromatogram for CA-01 showed maximum retention time of 2019 at 230nm (Fig 3).

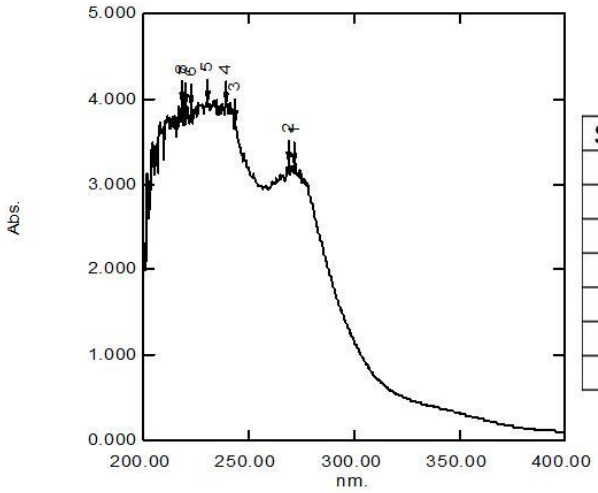


Detector A Ch1 230nm

Peak#	Ret. Time	Area	Height	Area %	HETP	Tailing Factor	Resolution	Theoretical Plate#
1	2.019	15937	1367	100.000	196.879	1.947	0.000	761.888
Total		15937	1367	100.000				

Fig 3. HPLC profile of the purified compound CA-01

Physical property profile of CA-01: The physical property study of bark extracted compound CA-01 revealed that the yellow coloured compound in amoophous powder is easily solubile with methanol, ethanol and slightly soluble in water at 200-202 ^{o}C melting point. The CA-01 compound was subjected for UV spectral studies showed the maximum absorbance of 3.983 at 272nm (Fig 4.).



Sl.no	Wavelength	Absorbance
1.	272.00	3.983
2.	269.20	3.959
3.	243.40	3.942
4.	230.20	3.923
5.	223.00	3.737
6.	220.40	3.265
7.	218.60	3.247

Fig 4. UV spectrometric absorbance profile of CA-01 at 272nm

IR Spectra analysis: The IR spectra of isolated compound CA-01 revealed the presence of function groups such as free OH (3749.07 cm^{-1}), C=C stretching (2360.43cm^{-1}) and other important functional groups(Fig 5 and Table 10).

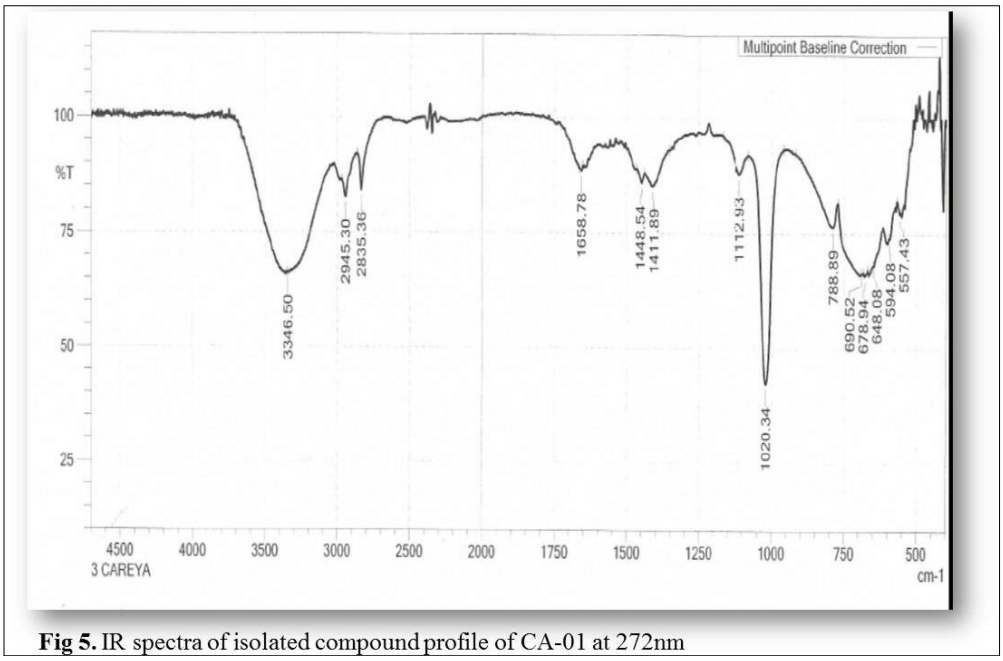


Fig 5. IR spectra of isolated compound profile of CA-01 at 272nm

The consolidated spectral data and physical properties is summarised in Table 10.

Table 10. The consolidated data of spectral studies of CA-01 compound.

	COMPOUND CA-01	INFERENCE
UV absorption(λ_{max})	272 nm	Conjugation as well as chromophore.
IR	3749.07 cm^{-1}	free -OH
	3352.89 cm^{-1}	OH group hydrogen bonded
	1683.01 cm^{-1}	alkyl stretch with C=C
	2360.43 cm^{-1}	C=C streching
	2945.39 cm^{-1}	C-H strech
	1869.80 cm^{-1}	C=O stretch
	1496.90 cm^{-1}	CH3 group
	699.14 cm^{-1}	Halogen group
Solubility	Ethanol, methanol and slightly in water	Polar in nature
Melting point	202.0^{0}C	-
Retention time (R_t)	2.019	Polar in nature
% purity	99.99%	_

Identification of compound and its structure: The isolated compound CA-01 was subjected for spectroscopic studies and structural elucidation by UV, IR. These studies result was compared to the values from literature and it possible suggested that compound is 2,4-dihydroxy-7-methynaphthalen-1(4H)-one with following structure.

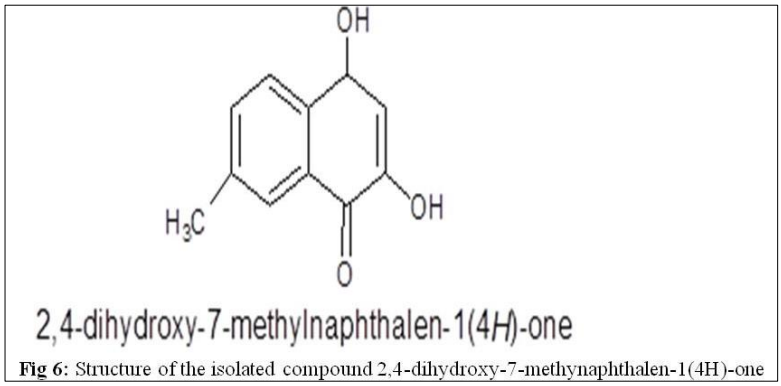


Fig 6: Structure of the isolated compound 2,4-dihydroxy-7-methynaphthalen-1(4H)-one

4. Conclusion

- The coarse powder of bark of *Careya arborea* subjected for successive extraction process by using soxhlet apparatus the highest amount of extract was obtained by using methanol.
- The methanolic extract of bark was used for ioptimization of solvent systems for TLC studies and isolation by using column chromatography. The mobile phase selected was n-butanol: glacial acetic acid: water (4:1:5) showed best pattern of band separation. The methanolic extract was packed into column and eluted with hexane: acetone to obtain 11 fractions. The 10^{th} fraction showed the three bands with similar chromatographic pattern by TLC identity tests. This fraction was subjected for preparative column for isolation and purification of compound. Purity of the isolated compound was determined by HPLC analysis.
- The isolated compound CA-01 was subjected for spectroscopic studies. The compound CA-01 showed the maximum absorbance at 272nm. The isolated compound was subjected to structural elucidation by UV, IR. All the above data was compared to the values from literature and it possible suggested that compound is 2, 4-dihydroxy-7-methynaphthalen-1(4H)-one with following structure.

Consent
It is no applicable.

Ethical Approval
It is no applicable.

Competing Interests
Authors have declared that no competing interests exist.

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