ISOLATION AND CHARACTERIZATION OF SELECTIVE HERBAL MARKERS FROM GARCINIA INDICA EXTRACT

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Abstract

As the market for herbal goods grows, it will be necessary to build trustworthy, cutting-edgetechnical interventions to find fake, mislabeled, and contaminated products. In this case, we have established cutting-edge technical ways to test the Garcinia indica (G. indica) which is a member of the Kokum-related Guttiferae family. It prevents obesity and functions as anantioxidant and digestive tonic, and treats liver conditions, sunstroke, cancer, and heartconditions. Garcinol, citric acid (CA), hydroxycitric acid (HCA), hydroxycitric acid lactone (HCAL), tannins, carbs, fiber, lipids, proteins, and anthocyanin pigments are some of thephytoconstituents discovered in it. Garcinol isolation by vacuum column chromatography andsize exclusion chromatography is performed after dichloromethane: methanol (1:1) extraction ofdried fruit rinds. Toluene, ethyl acetate, and acetic acid (7:3:0.2 v/v/v), used as the mobile phase,were used to develop the high-performance thin layer chromatography (HPTLC) method. Thehigh-performance liquid chromatography (HPLC) method was established using C_{18} (250mm x4.6mm, 5 m) as the stationary phase, 0.01M potassium dihydrogen phosphate (KH_2PO_4) as themobile phase A, and acetonitrile: 0.01M KH_2PO_4 (90:10 v/v) as the mobile phase B. Thesamples were then detected at 215nm and 276nm. Garcinol had a 0.69 retention factor (Rf) according to the HPTLC technique. HCAL, HCA, CA, and Garcinol all had HPLC retentiontimes that were, respectively, 2.941, 4.25, 8.666, and 18.467 minutes. This study may aid in theprevention of adulteration as well as quality assurance, standardization, and phytoconstituentidentification in G. indica formulations.

Keywords: Garcinia Indica extract, Herbal Markers, HPTLC, phytochemicals constituents

1. INTODUCTION

1.1 Introduction to *Garciniaindica*

Kokum derived from *G. indica*, a member of the Guttiferae family. The western ghats of maharashtra, goa, karnataka, and kerala are its typical habitat. And eastern India, which comprises the states of west bengal, assam, and the north eastern ill area. Between the months of march and may, the summer season exists. The tree consistently produces fruit during the warm months of march to may.

Kokum syrup, Kokum agal (Kokum juice concentrate), Kokum sarbat, Kokum solkdhi, Kokum amsul (dried salted rind), Kokum butter, and Kokum beverages are among the various products available on the market that contain kokum fruits, rinds, and fat. Rinsings are dried and stored so they can be used to manufacture reconstituted beverages during the off-season.

The fruit rind of *G. indica* has been utilized as a source of pink and purple food coloring, as it contains 2% to 3% water-soluble red pigments. The principal coloring components are the anthocyanin pigments cyanidin-3-glucoside and cyanidin-3-sambubioside, which are typically present in a ratio of 4:1. The variation in color tones of kokum fruits is due to the replacement of hydroxyl and methoxyl groups onto the anthocyanin structural skeletons. *G. indica's* radical scavenging capacity is determined by the 3' and 4'-OH in the B-ring with a saturated 2, 3-double bond, whereas the 3' and 4'-OH in the B-ring determine the radical scavenging capacity with a saturated 2, 3-double bond. The below**Figure 1**isthe key phytochemicals derived from *G. indica*, as well as their structures. [1]

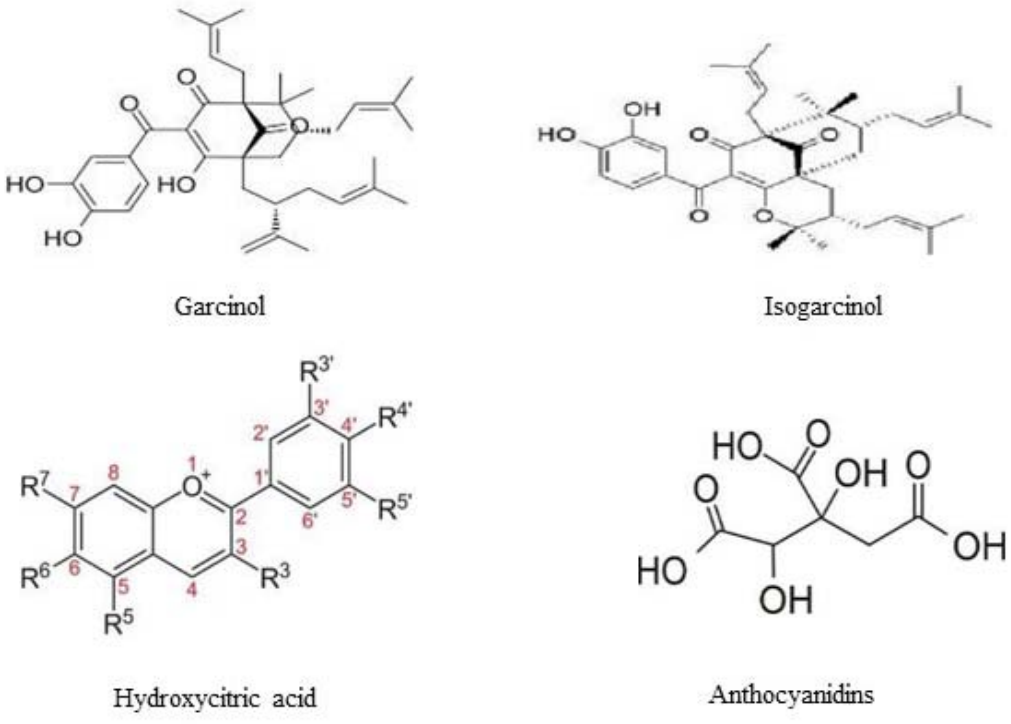

Figure. 1 Structure of chemical constituent present in kokum fruit rinds

1.1.1 Chemical Constitution

Kokum rind has three important chemical compounds: garcinol, hydroxycitric acid, and anthocyanin colors. Garcinol is a fat-soluble yellow pigment, while hydroxycitric acid is a chemical with established weight loss benefits. Kokum's anthocyanin pigment acts as a natural antioxidant. [2-5]

1.1.2 Traditional Uses

Garcinia Indica has many types of medicinal properties, it studies and investigated for the activities like, Anticancer activity, Antioxidant activity, Ant obesity activity, Antifungal activity and Antiulcer activity.[6]

2. OBJECTIVES

This study was planned to isolate the garcinol and HCL lactone from the Garcinia Indica by vacuum chromatography and characterization of garcinol by LC-MS, HPTLC and HPLC. Along with that to develop a single method to identify the selective herbal markers such as a HCA lactone, HCA, CA, Garcinol from the plant extract.

3. MATERIAL AND METHODS

3.1 Extract preparation and isolation of Garcinol from *G. indica*

3.1.1 Plant material

Fruitrindsof*G. indica*werecollectedfromlocal market in Ahmedabad.

3.1.2 Extraction from plant

Shade-dried pulverized *G. indica* fruit rinds (1 Kg) were prepared. Following this, the dried fruit rinds

were macerated in a mixture of dichloromethane and methanol (1:1) for three separate sessions totaling 24 hours at room temperature. The solvents were recovered using a rotary evaporator, and the extract was dried using a high-vacuum evaporator at 40 degrees Celsius to produce 150 grams. Put 10 grams' worth onto a petri dish, and then dry it in a 40 degrees Celsius high-vacuum oven. Place the dry yield in a separating funnel and fill it with a solvent, like n- Hexane. Acetic acid, ethyl: A mixture of methanol and water with a (1:1:1:1). To divide the organic phase into an upper and lower layer (aqueous phase). The TLC fingerprint for mobile-phase Garcinol is a Toluene: ethyleneaminoacetylene (EA: AA) mixture (7:3:0.2). The top layer was then concentrated in a 40 degree C high vacuum to produce 5 grams. Then, a vacuum column chromatography separation of the garcinol compound is performed.

3.1.3 EvaluationofConcentratebyThinLayerChromatography

Thin-layer chromatography (TLC) is a powerful tool in the scientific community. TLC is a rapid and inexpensive approach for determining how many different substances are present in a solution, finding the best solvent for flash column chromatography, and keeping tabs on how a reaction is going.

To establish how many chemicals are in *G. indica* extract, TLC was used with a variety of solvent/mobile phase combinations of decreasing polarity. Ethyl acetate/n-Hexane (9:1), ethyl acetate/n-hexane (9.5:0.5), ethyl acetate/n-hexane (9:1), ethyl acetate/n-hexane (8.5:1.5), Ethyl acetate/n-Hexane (8:2), Ethyl acetate/n-Hexane (7.5:2.5 (7:3). The results show that flash chromatography must use all of these solvent mixtures to successfully isolate garcinol from *G. indica* Extract.[8]

3.1.4 SeparationofextractbyVacuumColumnChromatography

Vacuum column chromatography is an excellent method for separating complex chemical mixtures into their individual components. The experiment was conducted using Aldrich Chemical Company-supplied silica gel (60-120 mesh) in a column of 60 cm in length and 3 cm in diameter. After suspending the mixture of around 5.0 g silica gel and 20 mL ethyl acetate, the ethyl acetate was extracted under vacuum using a high vacuum oven set to 40°C, and the *G. indica* extracts were fed into the column as a solid mixture with some silica. First, low-polarity pure hexane was used for the separation, followed by 1% ethyl acetate-containing hexane, then ethyl acetate at increasing concentrations up to 50%, then pure ethyl acetate, and lastly 100% methanol for the column flush. [9-10]

3.1.5 Size exclusion chromatography

Proteins and other macromolecules can be separated according to their molecular size using a technique called size-exclusion chromatography, commonly known as gel filtration chromatography. Size-exclusion chromatography is commonly utilized because to the diverse protein molecular weights present in biological tissues and extracts. The fundamental idea of size exclusion chromatography is that different sized solutes will be separated in different fractions of the column. Large molecules are eluted by the void volume (V0), while small molecules are eluted by the total volume (Vt). Now that garcinol has been successfully extracted from *G. indica*, we may move on to isolating other compounds. And HPTLC, HPLC, and HPLC all confirm the presence of garcinol. [11-13]

3.2 Extract preparation and Isolation of Hydroxycitric acid from *G.indica*

3.2.1 *Plantmaterial*

Commercially available in India, the acid is found in quantities of 20-30% in the dried fruit rinds of *Garcinia cambogia, Garcinia atroviridis,* and *G. indica*. Provide details on how hydroxycitric acid was extracted from *G. indica* here. Mix the rind of two hundred grams of *G. indica* fruit with six hundred milliliters of water and let it sit out at room temperature for 24 hours.

3.2.2 ExtractPreparation

After the extract has cooled, it is passed through several layers of muslin before being filtered through a Buchner funnel (Whatman No. 1 paper) and the residue is rinsed with water. The dark brown filtrate (originally 600 mL) is concentrated to around 100 mL by adding 200 mL of ethanol while being stirred over a water bath. Pectineus material is removed using centrifugation, followed by filtration. When adding 40% KOH to the acidic filtrate, it should be done so slowly and with careful stirring to maintain a constant pH of 7. Soaked in 500 mL of acetone in a 1 L flask overnight. Again, it was extracted in the same manner, this time with an equal amount of acetone. Acetone is evaporated off of the combined extracts using a rotary evaporator. The reddish-brown filtrate (80-90°C) is heated with activated charcoal (approximately 20 g) and reduced to a thick syrup in a water bath (light brown in color). The output is roughly 150 g. The material is extracted using 1 liter of ether (ten 300 mL sections), and the combined extracts are then dried over anhydrous sodium sulfate. Much of the pigment cannot be broken down by ether. After that, the extract is decolored with activated charcoal. After the ether has been distilled out, the extract material is dried off by heating it in a thin layer on a water bath for 10 to 15 minutes. So, having a pure white color. About 80 grams can be harvested.[14-15]

3.2.3 Purificationofisolatedconstituent

After obtaining lactone using either of the aforementioned methods, further purification is accomplished by repeatedly extracting it with diethyl ether (1g/20 mL). An equivalent volume of dry chloroform (chloroform passed from anhydrous sodium sulfate) is added to the concentrated substance soluble in diethyl ether and the mixture is stirred. When left exposed to air, the lactone crystallizes. After that, it was gathered and dried in a vacuum oven.However, HPLC validation showed that only 60% of the HCA was recovered using these isolation procedures.

3.2.4 IdentificationofisolatedcompoundAPI

HPTLC, LC-MS, and HPLC were all utilized in the identification of Garcinol. There were six visualizations of the datawhich are mentioned in result part. Melting point analysis and high-performance liquid chromatography were utilized to positively identify hydroxy citric acid lactone and hydroxy citric acid. In result part mention the data. The melting point, FTIR, and ultraviolet spectrum were used to positively identify citric acid.

3.3 PreparationsofsolutionforHPLC andHPTLCmethod

3.3.1 StandardStockSolution(SSS)

An accurately weighed 10 mg of Garcinol was placed in a 10 mL volumetric flask, then roughly 10 mL of methanol (HPLC) was added and sonicated for five minutes to dissolve adequately, yielding a standard stock solution of isolated garcinol (1000 g/mL). The final volume of the standard stock solution, 1000 g/mL, was adjusted to the correct level using the same diluent.

3.3.2 Preparation of 0.01M KH_2PO_4

Weigh 680 mg of KH_2PO_4 and put it in 500 mL of water. Then, sonicate it for 3 minutes. Use orthophosphoric acid to set the PH to 2.50 after measuring it. Then, a vacuum filter with 0.45 membrane filter paper was used to filter the solution that had been made.

3.3.3 Preparation of sample solution

G. indica fruit was bought from local market in Ahmedabad. The fruit rinds, which weighed 5 gm, were put in a 50 mL volumetric flask and diluted with water and MEOH to make 100000 g/mL. (Milli- Q and HPLC). Then, 0.2 mL of this solution was taken out and put into a 10 mL volumetric flask, where it was diluted with diluent to make 2000 g/mL.

3.4 RP-HPLCmethoddevelopment

3.4.1 Establishmentoftheoptimumconditionsof themethod

The HPLC method is chosen based on the type of sample (ionic, non-ionic, or neutral molecule), its molecular weight, and how well it dissolves. Reversed phase chromatography can be used to study the parts used in this study because they are both polar (HCA, CA) and nonpolar (Garcinol). Reversed phase chromatography is not only easy and convenient, but it is also more effective, stable, and repeatable. The C18 column is less polar than the C4 column and the C8 column. For better separation of the three analytes, a Waters Sunfire C18 250mm x 4.6mm column with 5 m particle packing was chosen. Gradient mode because it makes it easy to tell the peaks apart.

3.4.2 Selection of wavelength

The wavelength used to detect in an HPLC method that uses UV light showed how sensitive the method was. The best wavelength is the one that gives the best response when drugs are being looked for. In this study, drug solutions of Garcinol, HCA, and CA (1000 ppm) were made. The UV range of 190-400 nm showed that this medicine solution absorbed the lightest. The spectrum of a 1000 ppm solution shown the wavelengths where Garcinol (276 nm), HCA (215 nm), and CA (215 nm) are the strongest. Since the drug absorbs well at 276 and 215 nm for Garcinol and HCA, CA, respectively. For detection, these wavelengths were chosen.

3.4.3 Selection of Column

A review of the literature led to the choice of the C18 column for the trial. The study was first done on a C18 column, which helps separate things better. So, the C18 column was chosen to be looked at.

3.4.4 Selection of Mobile phase

Varying mobile phases, including methanol, water, acetonitrile, and KH_2PO_4 buffer, were evaluated in different quantities, volumes, and flow rates in a series of experiments. Based on numerous tests, the 0.01 M KH_2PO_4 (A) and ACN: 0.01 M KH_2PO_4 (90:10) (B) gradient modes, at a flow rate of 1.2 mL/min and a pH of 2.50, give superior peak form, theoretical plate, and asymmetry. We attempted various flow rates and proportions of mobile phase in isocratic and gradient modes, but the peaks of three analyzers could not be separated. To solve this issue and assure complete resolution, gradient elution was utilized, beginning with a low fraction of mobile phase B (ACN: 0.01 M KH_2PO_4 (90:10)). Several gradient programs were tested, but the greatest balance between adequate resolution, tolerable retention times, and acceptable peak shape was found with the gradient program utilizing solvent (A) 0.01 M KH_2PO_4 and mobile phase B with ACN: 0.01 M KH_2PO_4 (90:10). Various mobile phase check and mention in **Table 1**.

Table1.Initial HPLCOptimizedchromatographiccondition and mobile phase trials

Parameters	Chromatographiccondition
Chromatographicmode	Reversedphase
Modeofelution	Gradient
Column	WatersC_{18}(250mmx4.6mm,5µm)
Flowrate	1.2 mL/min
Columnoven temp.	25°C
Run time	30 min.
Injectionvolume	10µl

Detectionwavelength	210and 276 nm		
Mobilephase	Buffer0.01MKH_2PO_4		
	Time(min.)	A(Buffer)	B (ACN: Buffer90:10)
	0	100	0.0
	3	100	0.0
	6	90	10
	8	90	10
	12	0.0	100
	25	0.0	100
	30	100	0.0

4.4.1 Flow rateoptimization

Initiallyworkwasperformedwith 1.0mL/min, and1.2mL/minwerealsotried.atFlowrate of 1.2 mL/min was finalized for analysis as it gave reasonable analysis time andgoodseparation.

4. RESULT AND DISCUSSION

4.1 Identification ofDrug

UV spectra of *G.indica* extract in methanol demonstrate that the medication absorbs significantly at 224 and 282 nm for Garcinol, and HCA lactone -HCA, respectively. Consequently, the wavelengths 224 and 282 nm were chosen for detection as shown in **Figure 2.**

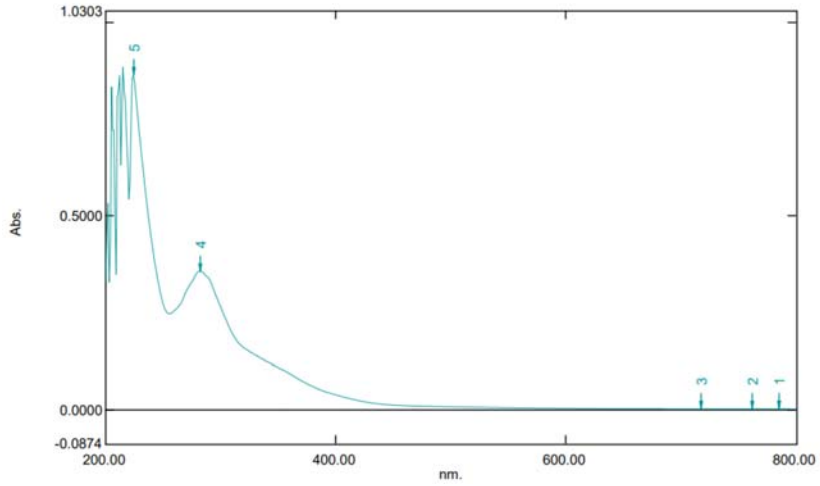

Figure. 2 UV spectrum of Garcinia indica extract

4.2 Determination of Solubility

According to the literature review and the nature of the constituents, Garcinol was found to be soluble in methanol and HCA lactone, HCA, and CA were found to be soluble in water. Different trials in water and methanol in different ratios were conducted, and the water: methanol (70:30) ratio was chosen based on UV absorbance.From the trials, HCAL, HCA and CA were soluble in in 100:0 (water:methanol) and garcinol was insoluble, another trial of 50:50 ration of water: methanol have shown the solubilty of all the constituents but peak sharpness was not achieved. While, 70:30 ratio of water:methanol have shown solubility with sharp peak.

4.3 Confirmation of isolated Garcinol

4.3.1 *IdentificationbyHPTLC*

Extract of *G. indica* in water: methanol (70:30) 10 µl spot on TLC, it given 7different band of compound in mobile phase is toluene: EA: AA (7:3:0.2). The rf value was found 0.69 of garcinol. (**Figure.3**)The band is then dissolved in methanol, and recollected using mass interference. Then confirmation of mass of garcinol by LC-MS.[8]

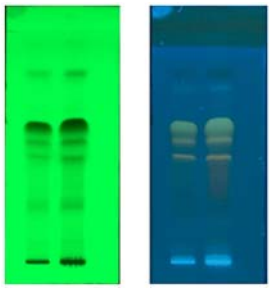

Figure. 3 HPTLC chromatogram of garcinia indica extract at 254 nm and 366 nm

Isolated Garcinol 10µl, 1000 ppm in mobile phase toluene: EA: AA (7:3:0.2)were tried but band having tailing effect. Then various combination of mobilephase was tried. In toluene: methanol: AA (7:3:0.2 v/v/v) band was sharp atsatisfactoryRf which is 0.76 andwas selectedas mobilephasein the method.**(Figure. 4)**

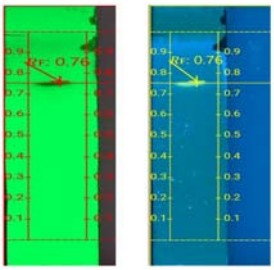

Figure. 4 HPTLC chromatogram of isolated Garcinol at 254 nm and 366 nm

4.3.2 Identification of Garcinol by LC-MS

The molecular weight of the isolated garcinol was confired by the LC-MS technique. 601.0 gm/mol of molecular wt. was observed by LC-MS and it was found nearer to the theoretical molecular wt. about 602.8 gm/mol.**(Figure. 5)**

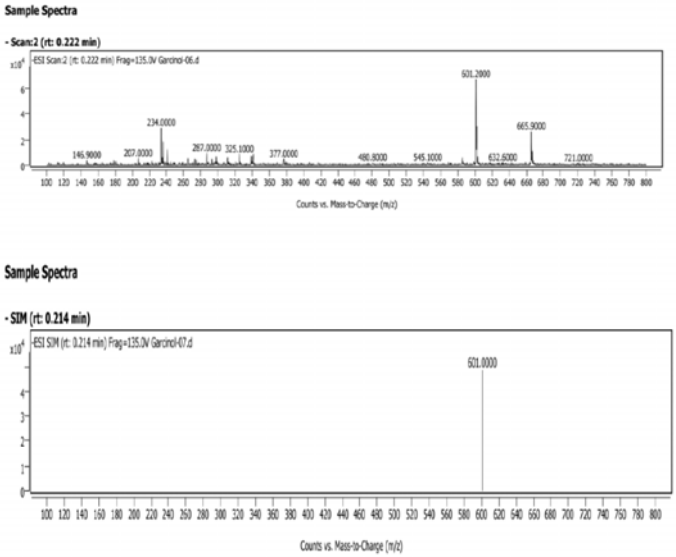

Figure. 5 LC-MS chromatograms of extract isolated Garcinol

4.3.3 Identification ofGarcinolbyHPLC

A number of mobile phase combination were tried**.** But isolated garcinol wasgiven sharp peak at RT 18.46 min, in mobile phase such as a ACN: 0.01MKH_2PO_4(90:10)(PH 2.50). With other chromatographic conditions like flow rate of 1.0mL/min, peak area of 100%, tailing effect of 1.267 and Plates USP about 11181.041.**(Figure. 6)**

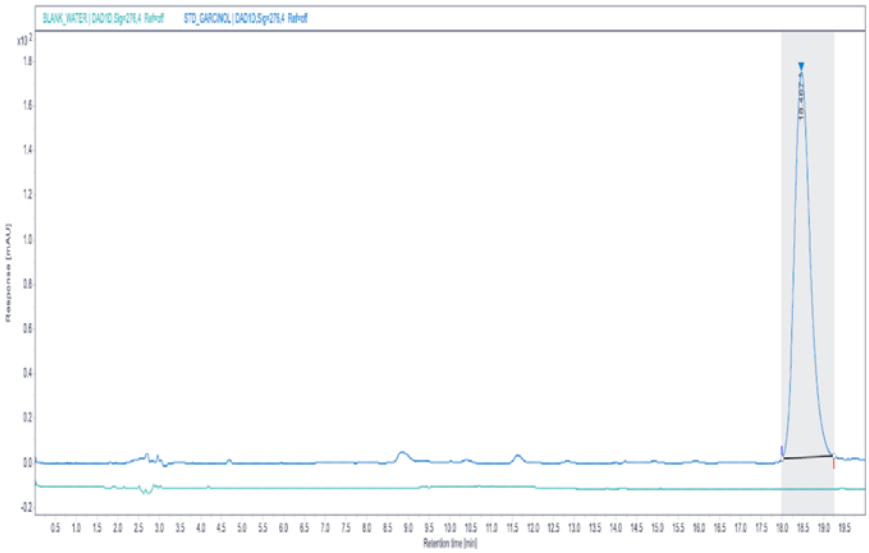

Figure. 6 HPLC chromatogram of isolated Garcinol

4.4 Identification of HCA lactone and HCA

4.4.1 *Identification of isolated HCA lactone, HCA by Melting point*

Thesamplewastakenincapillaryandplaceintotheheatedliquidparaffinbath. Observed melting point was found around 190-195 °C, which is nearer to >188 °C of reported value.

4.4.2 Identification of isolated HCA lactone, HCA byUVspectroscopy

UV spectra of HCA lactone or HCA in water, it is revealed that at 220 nm, drughavesignificanceabsorbance.Hence,220nmwavelengthwasselected for detection.**(Figure. 7)**

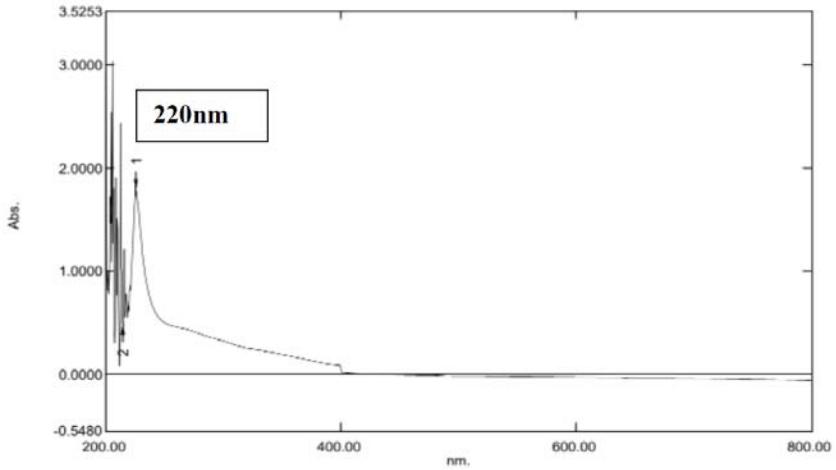

Figure. 7 HPLC chromatogram of isolated Garcinol

4.4.3 Identification of isolated HCA lactone,HCAbyHPLC

A variety of mobile phase combinations were tried, but extracted HCA lactone andHCA shows good peaks at RT 2.971 and 4.25 min, respectively, in a 0.01M KH_2PO_4(90:10)(PH 4.75)mobilephase. (**Figure. 8)**

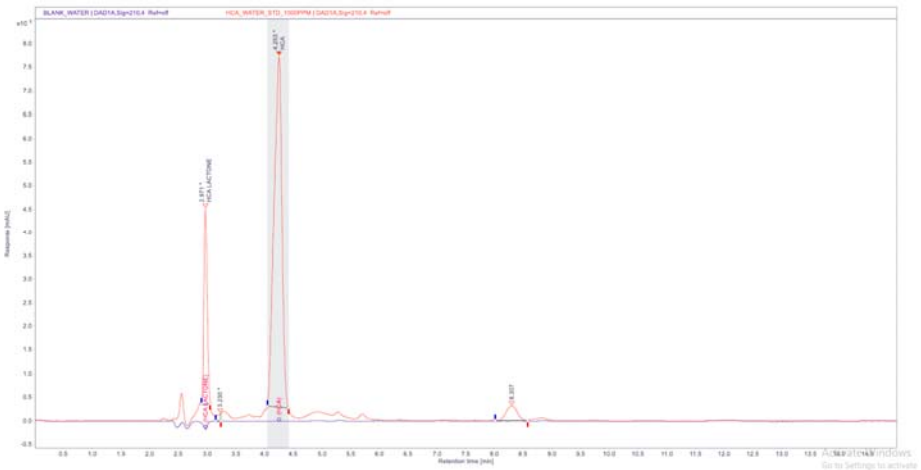

Figure. 8 HPLC chromatogram of isolated HCA lactone and HCA

4.5 Identification of citric acid

4.5.1 IdentificationbyUVspectroscopyforcitricacid

From UV spectra of citric acid in water, it is revealed that at 235 nm, drug have significant absorbance. Hence, 235nmwavelengthwasselected fordetection.(**Figure. 9)**

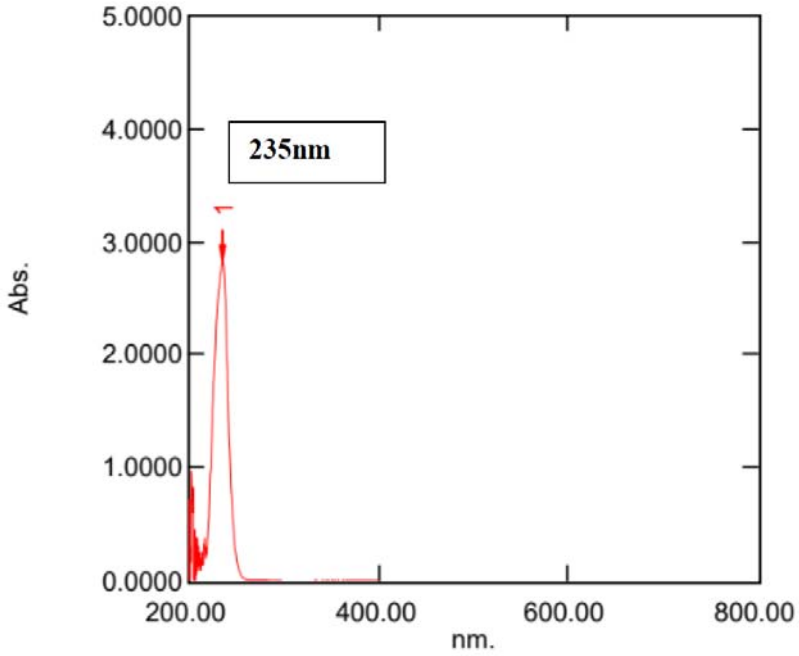

Figure. 9 UV spectra of citric acid

4.5.2 Identification by IR for citric acid

Citric acid standard was scanned in the region of 4000-400 cm-1 in FT-IR andobtainedIR

spectrumwas compared withthereferencespectrum. All the predicted standard frequencies were observed and confirmed the functional groups for citric acid. (**Figure. 10**)

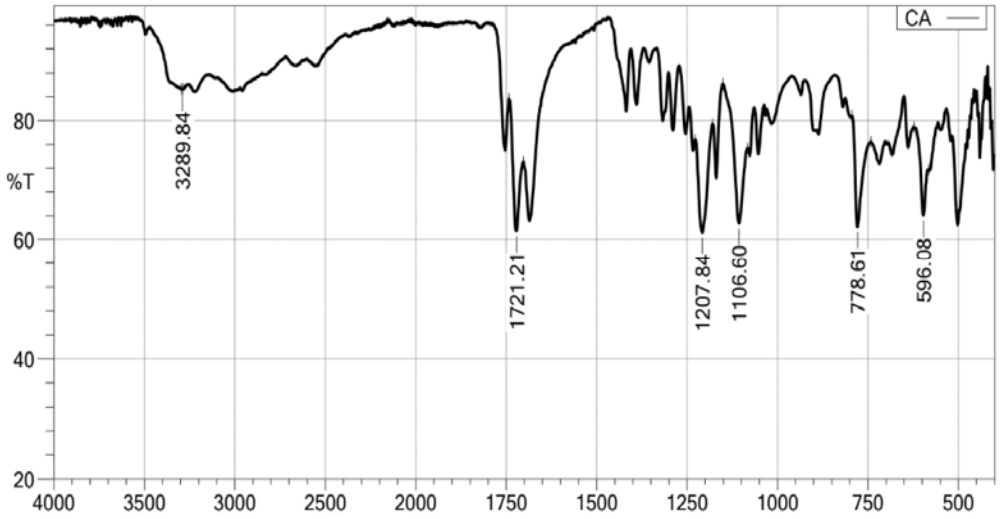

Figure. 10 IR spectra of citric acid

4.5.3 Determination of Melting point for citric acid

The sample was taken in capillary and place into the heated liquid paraffin bath.Observedthe meltingpoint which is 159-160°Cand comparedwith thereference which is 153°C.

4.5.4 Optimized chromatographic condition

Final HPLC chromatographic conditions for the detection of targeted constituents. Which found almost similar to the initially optimized HPLC method. Which can be used to detect the targeted molecules from any marketed herbal formulation.

Table2.Final HPLCOptimizedchromatographiccondition

Parameters	**Chromatographiccondition**
Chromatographicmode	Reversedphase
Modeofelution	Gradient
Column	WatersC18(250mmx4.6mm,5µm)
Flow rate	1.2 mL/min
Columnoventemp.	25°C
Runtime	30 min.
Injectionvolume	10µl
Detectionwavelength	210and 276 nm

5. CONCLUSION

HPTLC analysis confirmed the presence of garcinol in a *G. indica* extract. Utilizing mass interference and mass spectroscopy, scrape the band. Garcinol was then recovered from *G. indica* extract utilizing chromatography techniques such as vacuum column chromatography and size exclusion chromatography. Garcinol was validated through the use of HPTLC, HPLC, and LC-MS methods.

According to a review article, the *G. indica* extract contains HCAL and HCA. After isolating the ingredient and validating it with HPLC, we were able to separate HCAL and HCA from *G. indica* extract, however we were unable to separate HCAL and HCA using this isolation strategy. The developed HPTLC, HPLC, and LC-MS techniques for the confirmation of the isolated compound Garcinol, as well as the HPLC, IR techniques for the confirmation of the isolated compound. This designed and developed approach can be used to detect and identify the proposed target constituents from the marketed formulations to check the authenticity of the product.

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