RESEARCH ARTICLE

Isolation and Identification of Bioactive Chlorogenic Acid from Ethanol Fraction of *Cordia subcordata*: HPLC, HPTLC, FTIR, and LC-MS Analysis

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Received: 05th March, 2024; Revised: 26th June, 2024; Accepted: 13th July, 2024; Available Online: 31st August, 2024

ABSTRACT

Cordia subcordata was subjected for soxhlet extraction using soxhlet apparatus by hydroalcoholic solvent (Ethanol:water; 70:30). The obtained crude extract was subjected to organoleptic properties. The extract was dark greenish or greenish-black in color. The odor of the extract was strong like tamarind. The extract taste was bitter mixed with pungent and the texture was sticky solid. The yield of the extract was 9.1%. It was observed that crude extract contains carbohydrates, reducing sugars, monosaccharides, proteins, amino acids, fats and oil, steroids, cardiac glycosides, saponin glycosides, alkaloids, tannins and phenolic compounds, and flavonoids. The presence of many phytochemicals in the extract signifies its substantial contribution to various disorders. The crude extract was subjected to column fractionation using various solvents. For column fractionation, we used polar solvents and analyzed the resulting fractions using thin-layer chromatography (TLC) to determine the presence of individual or multiple compounds. From TLC analysis, it was observed that the ethanol fraction contains a single compound and therefore same fraction was subjected for HPLC, HPTLC, FTIR, and LC-MS analysis. From HPLC analysis, it was clearly identified that this ethanolic fraction contains chlorogenic acid and it displayed a peak at 8.428 minutes. The HPTLC analysis revealed the presence of chlorogenic acid. From HPTLC analysis, chlorogenic acid was identified at 0.65 Rf value. From the present investigation, we concluded that this developed method can be used for the isolation of chlorogenic acid from *C. subcordata*.

Keywords: Isolation, Fractionation, Phytochemical screening, Hydroalcoholic extraction, HPLC.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.3.07

How to cite this article: Patil VP, Khulbe P. Isolation and Identification of Bioactive Chlorogenic Acid from Ethanol Fraction of *Cordia subcordata*: HPLC, HPTLC, FTIR, and LC-MS Analysis. International Journal of Pharmaceutical Quality Assurance. 2024;15(3):1144-1150.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Chlorogenic acid, a sizable polyphenolic compound, is widely available in many plants, coffee beans in particular. It has good number of health properties which are beneficial. Its antioxidant properties, which are very potent, can curb the negative effects of the free radicals that exist in the body by neutralizing them. This action also limits the oxidative stress and inflammation in the body. Research indicates that chlorogenic acid could play a role in decreasing blood pressure and enhancing cardiovascular health by relaxing the blood vessels and preventing the formation of cholesterol plaques in the arteries.[1,2]

In addition to that, chlorogenic acid shows anti-diabetic potential that regulates the metabolism of glucose and insulin sensitivity which further makes it a powerful natural compound in type 2 diabetes control. Besides, these chemicals has antimicrobial activities that help to fight against different pathogens, and the neuroprotective effects may be used to prevent age-related cognitive decline and neurodegenerative diseases like Alzheimer's and Parkinson's. Also, the fact that chlorogenic acid has anti-inflammatory properties is associated with a decrease in the chance of chronic diseases like cancer and arthritis. The efficiency of the compound in the regulation of lipid metabolism and promotion of weight loss should be considered as another potential influence in the context of the obesity management battle. Therefore, it can be concluded that chlorogenic acid due to its multidimensional health benefits, is a very valuable bioactive compound having therapeutic importance in multiple disorders[3-5] a group of hydroxycinnamates, are generally abundant in everyday foods and beverages, most prominently in certain coffee drinks. Among them, the chlorogenic acid (CGA.

The Beach Cordia (*Cordia subcordata*) is a tree belonging to the family *Boraginaceae* with the typical tropical habitus.

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Native to the Indian and Pacific Coastal regions, this grass is known for its hardiness and ability to live in sandy soils and coastal conditions, therefore making it a popular choice in beach landscaping and especially controlling beach erosion. The tallest tree can grow up to 15 to 20 m in height with glossy, elliptical leaves and clusters of small white flowers that bloom all year round. However, *C. subcordata* appears to have both ornament value and cultural value as the wood is traditionally used in Pacific island culture for carving, canoe building and timber. The tree's bark and leaves have been utilized in traditional medicine for decades to harvest the active ingredient that gives anti-inflammatory, analgesic, and antimicrobial effects. Furthermore, the several components of the plant, including the fruits and seeds, are used in traditional cuisine and as food sources for wild animals. Although *C. subcordata* is a traditional species in use by humans and is also a cultural symbol, this species is still threatened with habitat loss and overexploitation, and therefore, it is imperative to implement conservation measures to protect such ecologically valuable coastal species[6,7] belonging to the Boraginaceae family, have strong resistance and have adapted to their habitat on a tropical coral island in China, but the lack of genome information regarding its genetic background is unclear. In this study, the genome was assembled using both short/long whole genome sequencing reads and Hi–C reads. The assembled genome was 475.3 Mb, with 468.7 Mb (99.22%. In the present study, considering the importance of chlorogenic acid, we have isolated it from the ethanolic extract of *C. subcordata*.

MATERIAL AND METHODS

Collection of Plant Material and Authentication

C. subcordata herb was collected in the month of July 2022 from local area of Tirupati, Dist-Tirupati, State-Andhra Pradesh, India. From the collected plant material, the herbarium was prepared and authenticated by Dr. K. Madhava Cheety, Assistant Profesor, Botanical Department of Sri Venkateswara University, Tirupati-517 502, Andhra Pradesh, India. A voucher specimen no. 0559 was deposited. After collecting the authentication certificate, the extraction procedure was performed.

Soxhlet Extraction using Hydroalcoholic Solvent

For extraction, the whole plant material of *C. subcordata* was gathered and subsequently rinsed in distilled water to decrease the presence of dust or any other particles, and the plant was naturally dried at room temperature for a week. One should ensure it dries in room temperature in order not to lose some of the volatile Phytoconstituents. About 500 grams of dried plant material was ground into a fine powder for further extraction. The resulting powder was subjected for Soxhlet extraction using Soxhlet apparatus by hydroalcoholic solvent (Ethanol:water; 70:30). The powder material was filled in the thimble tube and the solvent was poured gradually about 200-300 mL until the completion of one syphon cycle. Then at least 72 hours were devoted to the extraction process, during which time up to 100 to 120 syphon cycles were completed. The dark green hue of the solvent indicated that the greatest amount of phytoconstituents had been extracted from the sample. The extraction was continued till it showed very light green color in the syphon tube. The solvent was then evaporated off from the obtained extract at room temperature[8-10] identified and characterized. Direct and sequential soxhlet extraction and its concentrated fractions were subjected to thin layer chromatography and high performance thin layer chromatography. The results showed that maximum yield of the flavonoid (6.53 g. The obtained extract was subjected for organoleptic investigation and phytochemical screening as per the reported methods.[11-13]

Isolation and identification of Chlorogenic Acid

Fractionation by column chromatography and TLC analysis

The extract (ethanol:water; 70:30) was subjected to column fractionation using various solvents. It is evident that we have used a polar solvent for extraction, indicating that all the compounds being extracted possess a polar character. For column fractionation, we used polar solvents and analyzed the resulting fractions using TLC to determine the presence of individual or multiple compounds. The fractionation generally depends on the polarity of the solvent system; therefore, we have tried different kind of solvents with different polarity. The fractionation was done till the complete vanishing of extract sample was kept on the top of the column. A column with a sintered disc of 300 mL capacity with 18 mm bore size has been used. The slurry of silica gel (mesh size: 60-120) was prepared in water and it was poured in the column. The cotton bolus was kept at the top of the column, and the extract sample of about 5 to 8 g was kept on the top and again, one cotton bolus kept over it to avoid unequal distribution of extract. The obtained fractions were subjected for TLC analysis to check for the number of chemical compounds present in the fraction. The solvent system used for TLC was benzene:ethyle acetate: ethanol (4:4:2).

HPLC analysis of selected fraction from column chromatography

The obtained fraction was subjected for HPLC analysis to identify the compound present in it. The different trials were run to optimize the method. The trials and chromatograms are given below:

Trial I

Different chromatographic conditions were used on the tested samples for separation and determination of chlorogenic acid. For HPLC analysis, the samples were analyzed on an Agilent® 1100 LC system. The development of the system was accomplished by redesigning all the dead volume areas and enhancing the functions of the device, such as higher UV scan rate, modification of the injector needle seat, incorporation of which was new red PEEKsil™ Tubing (SGE) in the system and also the use of the semi-micro flow cell. The method utilized a Luna® 5 µm C_{18}(2) 250 x 4.6 mm fully porous HPLC column.

From the initial scan using solvent system water: acetonitrile (80:20) and scanned in PDA (200–400 nm), it was observed that this extract might contain chlorogenic acid and therefore, further we tried to develop the method which can determine the same. During method development, tailing and peak fronting were observed. So, we are switching to other trials to get rid of the aforementioned problem.

Trial II

In II trial, the identification of chlorogenic acid in the extracts was successful with the mobile phase, prepared with a mix of o-phosphoric acid with still water (0. 2%) and acetonitrile and a supelcosil LC 18 column, 250 x 4. 6 mm 5µm at a flow rate of 1. 2 mL/min at 25°C. The mobile phase was prepared with 0. 2% phosphoric acid in water (A), acetonitrile (B) and in gradient elution: At first, for the first 0 min the mobile phase was A–B (94:6, v/v), then for the period of 0 to 25 minutes there will be a linear change from A–B (94:6, v/v) to A–B (70:30); 25 to 30 minutes The simple linear gradient elution is from A–B (70:30). Samples chromatograms were taken using UVDAD Detector in total comparison wavelength of 330 nm. Measuring of the detector responses was done as the peak area. Regarding the procedure of injection, it was 10 µL. It was in the range of 0.5 to 1 mL/min At this flow rate the consumption of 15 litres of liquid and was determined by the flow rate of 1. 2 mL/min there was more of a retention time for chlorogenic that was found to be 8. 68 min. The peak of absorbance for chlorogenic acid sample was obtained after 330 nm and the data taken at this wavelength are used for analysis. In conclusion, in addition to the chlorogenic acid content of *C. subcordata*, the other constituents show more response at 330nm.

Trial III

After several trials we approached this HPLC condition using Inertsil ODS-SP Column (5 µm, 150 × 3.0 mm I.D.) and mobile phase Eluent: A) CH_3OH B) 5 mM in KH_2PO_4 (pH 2.5, H_3PO_4) buffer A/B = 30/70, v/v the optimum Flow rate was determined at 0.4 mL/min and column temp set at 40 °C. As in the above trials at 330nm, the response of other Cordia constituents is more. By keeping this in mind, the detection wavelength was selected at 280 nm (PDA 200 – 400 nm) and Inj. Vol. It was 10 µL. With this trial we found that the chlorogenic acid was well separated. In addition, no peak tailing and shoulder appeared during the development. Additionally, the run time was set for more than 15-20 min, in which all the phytoconstituent eluted.

HPTLC analysis

Software used Vision Cat 3.0. The standard operating procedure was employed to develop HPTLC, these include the following steps:

HPTLC is performed using 20×10 cm TLC plate coated with silica gel 60 F254. The following are the common steps incorporated for HPTLC development. The twin trough chamber was used for 20x10 cm plates of extract. A filter paper of focal size was fitted with the twin trough of the chamber. In the case of the rear trough, 20 mL of the developing solvent was piped over the filter paper so that it covered the entire area of the plate. To ensure that the developing solvent fills the tray adequately enough to have a depth of about 5 mm, a sufficient amount of the developing solvent was poured into the front trough so that the change was made and the lid closed to allow adequate saturation for 20 minutes. The band length of the sample is 8 mm and there are 8 tracks. The application position of the sample is at coordinates X:Y 15:8 mm. The distance between tracks is automatically set to a minimum of 11 mm. The sample solvent used is ethanol and the sample analysis type is *C. subcordata* ethanolic column fraction. The automatic chamber settings used include enabling pre-drying, and saturating with filter for at least 20 minutes. In order to control humidity, it is recommended to use MgCl2 for a duration of 10 minutes. The migration distance should be 70 mm. For drying, 5 minutes was dedicated with 10 mL of developing solvent and 25 mL of saturation solvent.

FTIR and LC-MS analysis

The obtained fraction was subjected for FTIR and LC-MS analysis to confirm the presence of chlorogenic acid. Amongst the techniques used in the identification of the herbal drugs FTIR and LC-MS are very critical. FTIR-based spectral analysis reflects the molecular fingerprint of the compound by measuring the infrared energy level of the sample, which helps to identify the functional group and structural properties present in herbal extract and on the other hand LC–MS, been a combination of LC and MS, which allows for identification and quantification of particular bioactive constituents present in the plant. Combined, these methods bring into sophisticated profiling and authentication of the herbal drugs, thereby helping in modern standardization, conventional quality control as well as likely therapeutic agents' discovery. Their complementary nature improves the ability to perform analysis on herbal drugs in a most accurate and reliable manner.

RESULTS AND DISCUSSION

Organoleptic Analysis of Crude Extract

C. subcordata was extracted using a hydroalcoholic solvent. The obtained crude extract was subjected for organoleptic properties. The extract was dark greenish or greenish-black in color. The odor of the extract was strong like tamarind. The extract taste was bitter mixed with pungent and the texture was sticky solid. The yield of the extract was 9.1%. Hydroalcoholic solvent extraction is an important technique of extracting phytoconstituents or secondary metabolites present in plant materials. With a water and alcohol combination, the extraction processes allow for the extraction of numerous polar and non-polar bioactive compounds in the complex matrix present in plant extracts. The use of this dual-solvent system increases the amount of extraction as well as the overall extraction yields hitherto unattainable, which include alkaloids, flavonoids and tannins. Also, cyclohexanol assists in maintaining the quality and steadiness of vulnerable molecules while preventing the denaturation of certain polymers. Hydroalcoholic extraction can also be considered relatively simple and inexpensive and

Table 1: The solvents used for fractionation and other details

Fraction No.	*Solvent*	*Run time (hours)*	*Fraction quantity (mL)*	*Color*
01	Water	3–4	23	Light yellow
02	Hot water	2–3	34	Yellowish green
03	Ethanol	4	26	Greenish brown
04	Ethyl acetate	4–5	20	Light yellow

thus of broad applicability in extraction as well as formulation and standardization of herbal extracts for medicinal purposes.

Phytochemical Analysis of Crude Extract

Phytochemical screening can be described as analyzing, studying, extracting, and testing with different classes of phytoconstituents in the place with the aim to discovering new medications. It becomes possible to also extract the base active ingredients for further inspection and analysis. Given that it is easier and less expensive to start a drug discovery program with phytochemicals sourced from medicinal plants, it is important to have an initial phytochemical analysis, known as screening of phytochemicals. It was observed that crude extract contains carbohydrates, reducing sugars, monosaccharides, proteins, amino acids, fats and oil, steroids, cardiac glycosides, saponin glycosides, alkaloids, tannins and phenolic compounds, and flavonoids. The phytochemicals present in this extract suggest it plays a major role in various diseases.

Column Fractionation and TLC Analysis of Extract

Column chromatography share as part of the techniques used in the separation of compounds from herbal extracts. It enables the unequal distribution of the samples in the solution because the elements in the solution have different retention characteristics to the two phases involved. This makes it easier to isolate the pure form of the compounds of interest in high concentrations so as to be studied further. Classification that is accomplished through the use of columns is consequently instrumental in determining the presence of certain medicinal properties as well as guaranteeing the selectivity and potency of herbs. Also, it helps in the filtration process, the removal and separation of unwanted substances, and a boost in the efficiency of the alternative extraction process. The column fractionation details are depicted in Table 1. Each fraction was then subjected to TLC analysis to understand the presence of a number of compounds in each fraction. The TLC images, Rf values, and observation are tabulated in Table 2.

From the above analysis, it was observed that the ethanol fraction contains a single compound and therefore same fraction was subjected for HPLC, HPTLC, FTIR, and LCMS analysis.

HPLC Analysis

The obtained chromatograms from different trials are depicted in Figures 1 to 3.

The optimization process for the HPLC conditions involved several trials, ultimately leading to the selection of the Inertsil ODS-SP Column (5 µm, 150 × 3.0 mm I.D.) and a mobile phase of methanol (A) and a 5 mM KH_2PO_4 buffer (pH 2.5, with

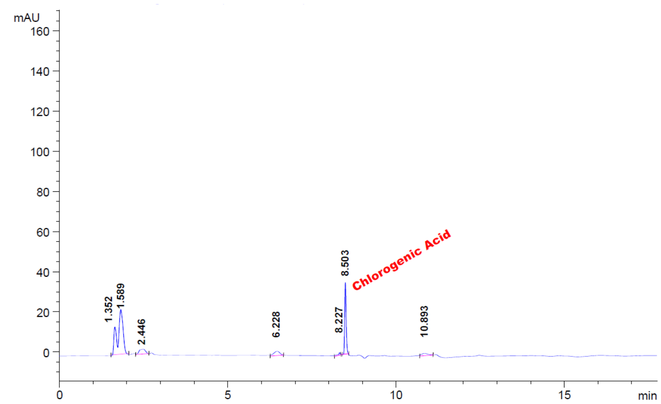


Figure 1: The chromatogram obtained through trial-I

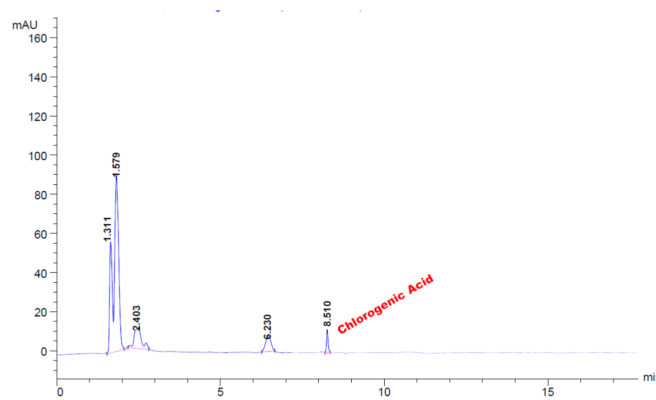


Figure 2: The chromatogram obtained through trial-II

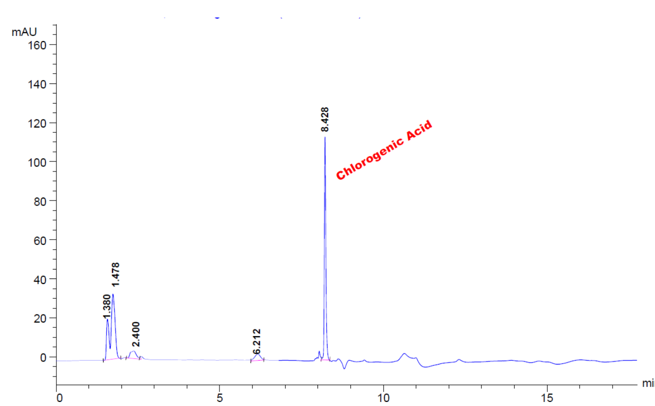
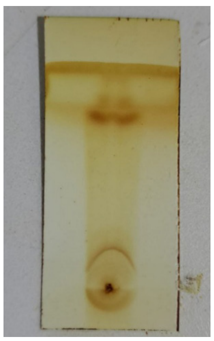
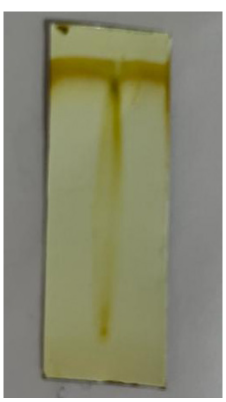
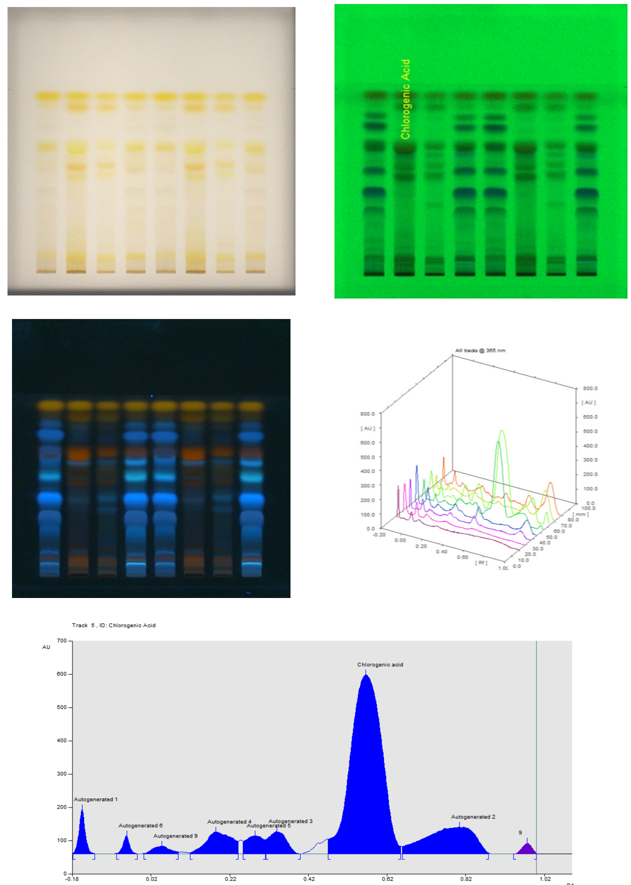


Figure 3: The chromatogram obtained through trial-III (Optimized)

Table 2: TLC analysis of obtained fractions

Fraction	*TLC*	*Rf value*	*Observation*
Water		0.92	Tailing with the splitting of peaks after run which may indicate the presence of multiple compounds.
Hot water		0.90	Tailing of peak with slight splitting: may be more than one compound.
Ethanol		0.91	Fine single peak indicating presence of single compound.
Ethyl acetate		0.93	Long tailing of peak indicating presence of multiple compounds.



Track 5, ID: Chlorogenic Acid

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.18 Rf	1.2 AU	-0.16 Rf	132.9 AU	13.09 %	-0.13 Rf	0.3 AU	1308.7 AU	3.07 %
2	-0.07 Rf	1.4 AU	-0.04 Rf	53.6 AU	5.28 %	-0.02 Rf	0.3 AU	566.2 AU	1.33 %
3	0.00 Rf	1.3 AU	0.05 Rf	23.4 AU	2.30 %	0.09 Rf	5.9 AU	658.2 AU	1.55 %
4	0.12 Rf	3.9 AU	0.18 Rf	66.2 AU	6.52 %	0.24 Rf	37.5 AU	2811.1 AU	6.60 %
5	0.25 Rf	38.4 AU	0.28 Rf	54.0 AU	5.32 %	0.31 Rf	42.6 AU	1534.8 AU	3.60 %
6	0.31 Rf	42.9 AU	0.34 Rf	66.8 AU	6.58 %	0.40 Rf	0.0 AU	1949.3 AU	4.58 %
7	0.47 Rf	44.2 AU	0.57 Rf	538.0 AU	52.99 %	0.65 Rf	22.8 AU	27244.9 AU	63.96 %
8	0.66 Rf	22.1 AU	0.80 Rf	80.5 AU	7.93 %	0.88 Rf	2.9 AU	6526.6 AU	15.32 %

Figure 4: HPTLC data of ethanolic column fraction

H_3PO_4) (B) in a ratio of 30:70 (v/v). The flow rate was optimized at 0.4 mL/min, and the temperature was set to 40°C. Despite previous trials showing higher responses for other constituents of Cordia at 330 nm, the detection wavelength was chosen as 280 nm, considering the separation of chlorogenic acid. A 10 µL injection volume was determined to be optimal. During this trial, chlorogenic acid was successfully separated with no peak tailing or shoulder observed during development. Furthermore, the run time was set for a duration exceeding 15 to 20 minutes to ensure elution of all phytoconstituents. This optimized HPLC condition provides a reliable method for the separation and quantification of chlorogenic acid in *C. subcordata* extracts, offering potential applications in pharmacological and phytochemical studies. From HPLC analysis, it was clearly identified that this ethanolic fraction contains chlorogenic acid and it displayed a peak at 8.428 min. The subsequent fraction then subjected for HPTLC analysis.

HPTLC Analysis

HPTLC plays a crucial role in qualitative analysis in view of the high sensitivity, resolution and the speed in which the

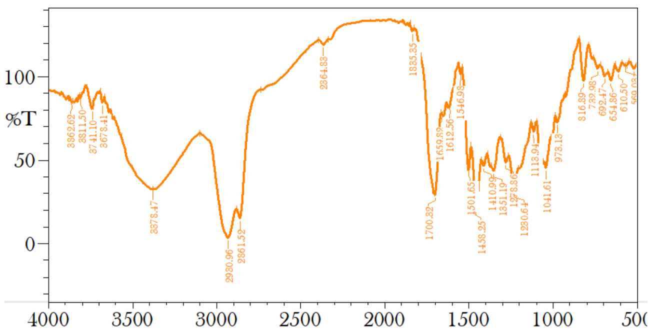


Figure 5: The FTIR graph of ethanolic column fraction

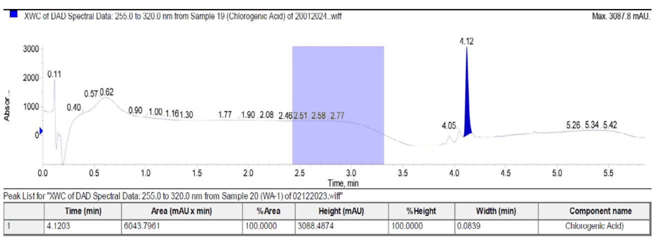


Figure 6: The LC-MS graph of ethanolic column fraction

method delivers its result. In this way, it involves the rapid and simultaneous testing of several samples and gives a visual representation of the parts. In routine, HPTLC is highly applicable in the identification and authentication of such complex formulations, for instance, crude natural products such as biological extracts to check on the presence and quality of active/ bioactive constituents. Its effectiveness in picking out the smallest component in a mixture makes it quite essential in issues to do with quality assurance and calibration. Similarly, HPTLC has attributes of being economical and the sample preparation is not elaborate, hence making it applicable in different sectors. The HPTLC plates and densitograms are given in Figure 4. The HPTLC analysis revealed the presence of chlorogenic acid. From HPTLC analysis, chlorogenic acid identified at 0.65 Rf value.

FTIR and LC-MS Analysis

From FTIR analysis, it is evident that the obtained fraction may contain chlorogenic acid as an active phytoconstituent (Figure 5). The presence of peaks at 2861.52 and 2930.96 cm^{-1} confirms the presence of carboxylic group. The presence of a sharp peak at 1700.32 cm^{-1} indicates the ester group in the structure. The broad peak at 3378.47 cm^{-1} shows the presence of phenolic –OH. The peaks from 610.50 to 816.89 cm^{-1} indicate the presence of an aromatic ring. The peak at 1041.61 cm^{-1} shows the presence of –C-O stretching of alcohols. Combined, FTIR graph indicates the functional groups of chlorogenic acid.

The LC-MS spectra of fraction is depicted in Figure 6, it denotes the presence of chlorogenic acid. It depicted peak at 4.12 minutes. Chlorogenic acid is a polyphenol, which is in existence in many plants and has a vital role in the management of ailments. This it inhibits the impacts of the free radicals, therefore lowering oxidative stress risks. The advantages of this compound are to enhance patients suffering from cardiovascular diseases because it aids in regulating lipid profile and pressure. Chlorogenic acid is also anti-inflammatory and anti-diabetic, help in maintaining correct blood glucose concentrations and improving insulin responsiveness. However, it is also found to have some benefits in its effectiveness as anti-obesity and anti-cancer since it is reported to be able to suppress the growth and metabolism of tumor as well as enhance the metabolism of fats. According to all these factors, chlorogenic acid has great versatile medicinal benefits that make it an essential component in today's pharmaceuticals.

CONCLUSION

C. subcordata was subjected for soxhlet extraction using Soxhlet apparatus by hydroalcoholic solvent (Ethanol:water; 70:30). The optimization process for the HPLC conditions involved several trials, ultimately leading to the selection of the Inertsil ODS-SP Column (5 µm, 150 × 3.0 mm I.D.) and a mobile phase methanol (A) and a 5 mM KH_2PO_4 buffer (pH 2.5, with H_3PO_4) (B) in a ratio of 30:70 (v/v). Despite previous trials showing higher responses for other constituents of Cordia at 330 nm, the detection wavelength was chosen as 280 nm, considering the separation of chlorogenic acid. A 10 µL injection volume was determined to be optimal. During this trial, chlorogenic acid was successfully separated with no peak tailing or shoulder observed during development. Furthermore, the run time was set for a duration exceeding 15-20 minutes to ensure elution of all phytoconstituents. This optimized HPLC condition provides a reliable method for the separation and quantification of chlorogenic acid in *C. subcordata* extracts, offering potential applications in pharmacological and phytochemical studies. From HPLC analysis, it was clearly identified that this ethanolic fraction contains chlorogenic acid and it displayed peak at 8.428 min. The HPTLC analysis revealed the presence of chlorogenic acid. From HPTLC analysis, chlorogenic acid was identified at 0.65 Rf value. Overall, these findings demonstrate the efficacy of the developed method for isolating and quantifying chlorogenic acid from *C. subcordata* extracts, highlighting its potential applications in pharmacological and phytochemical research.

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