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PHYTOCHEMICAL ANALYSIS OF INDIVIDUAL AND SYNERGISTIC EXTRACT FRACTIONATION OF CURCUMA CASSIA AND BERBERIS LYCEUM USING HPLC, GC-MS, FTIR AND NMR TECHNIQUE

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Abstract

Background: In the current study, we performed the phytochemical analysis of the individual and synergistic combination of Curcuma cassia, Berberis lyceum and their fractions to investigate their active constituents.
Objective: The objective of this study was to identify the major bioactive secondary metabolites present in Curcuma cassia and Berberis lyceum.
Methodology: The hydroalcholic extract of Curcuma cassia and Berberis lyceum were prepared by Soxhlet separately and synergically in an equal proportion. Crude synergistic extract was sequentially fractionated in n-hexane, chloroform and ethyl acetate in order of polarity. Qualitative phytochemical screening was done by standard tests, quantitative analysis of bioactive compounds by HPLC, GC-MS, FTIR, and NMR spectroscopy.
Results: We obtained 34.8% yield from synergistic extract and in fractions ethyl acetate fraction shows maximum yield 29.4%. We also identify the constituents present by qualitative phytochemical analysis. Through HPLC we identify many compounds, Cinnamic acid, coumarins; Tannins are the important one which we targeted. GC-MS also detect Cinnamaldehyde and coumarin, FTIR is used for detecting compounds and NMR analysis identify and quantify molecules type and their shifts.
Conclusion: Our study provided scientific support to researchers with strong background of the active compounds present in Curcuma cassia and Berberis lyceum.

Key words: Synergistic Extraction, Fractionation, phytochemical analysis, HPLC, GC-MS, FTIR and NMR.

1. INTRODUCTION:

The search for bioactive molecules from natural sources has a long-standing tradition in pharmaceutical research [1]. Most plants used in traditional medicine are rich in secondary metabolites that act on the same pathways as synthetic spasmolytic often with synergistic, multi mechanistic, and reduced risk profiles [2]. One such plant is Curcuma cassia, more widely known as Ceylon turmeric or aromatic turmeric or wild turmeric, is an aromatic, perennial herbaceous plant of the family Zingiberaceae [3]. It is very similar to Curcuma longa (common turmeric) but is phytochemicaly and botanically different. Recently, the plant has been receiving a lot of attention because of its abundance in bioactive compounds as well as due to its long history in traditional medicine systems with various diseases being treated with the help of this plant, especially inflammation-related conditions, infection issues, and gastrointestinal disorders[4]. This segue gives a detailed description of C. cassia with an emphasis on its botany, ethnomedicine uses, chemical compounds, and scientifically confirmed pharmacological uses[5]. The high secondary metabolic content in Curcuma cassia specifically the rhizomes, in the form of a large and diverse content of secondary metabolites, is given credit by the therapeutic efficacy of the medicinal plant. The main bioactive pigment curcumin is Diferuloylmethane, Demethoxycurcumin, Bisdemethoxycurcumin. The yellow-orange color and many of the antioxidant and anti-inflammatory properties of the plant are the result of these compounds[6]. Berberis lyceum Royle (syn. Berberis lyceum), commonly referred to as Kashmal, Zereshk, or Himalayan barberry evergreen shrub species in the family Berberidaceae[7]. Natively grown in the temperate and sub-alpine areas in the western Himalayas, it occurs in northern Pakistan, India (Jammu & Kashmir, Himachal Pradesh), Afghanistan and eastern Iran. It has over a century history of traditional medicine application in the plant to treat a myriads of diseases especially fever and liver ailments; urinary tract related infections; fouls on the human skin, as well as the stomach related diseases such as diarrhea and

dysentery[8]. Its gleaming yellow taproots and bark of the stem are source of several bioactive alkaloids which gives it value as a folk and Unani medicinal resource. The best characterized and highest profile alkaloid is berberine, an isoquinoline alkaloid which gives the root its characteristic yellow color [9]. The analysis of these components has been accomplished by application of advanced analytical procedures, including Gas Chromatography-Mass Spectrometry (GC-MS), High-Performance Liquid Chromatography (HPLC), and Fourier Transform Infrared Spectroscopy (FTIR) that help to profile and quantify the components and thus allow the standardization of the extracts to be used in pharmacological investigations. These plants phytochemicals tend to act synergistically in crude extracts, in turning out a wider field of the action than would be taken by isolated, synthetic medications [10]. As another example, an extract can inhibit Ca 2+ influx, increase K + efflux, decrease acetylcholine release and inhibit inflammation at the same time- an integrated approach to dealing with many facets of spastic diarrhea in one blend. Additionally, evaluation of the synergistic (i.e. combined) action of the two extracts could also lead to increased spasmolytic effect being observed at a reduced dose, with a potentially reduced likelihood of adverse effects and maximum benefit. It is consistent according to traditional polyherbal formulations principles which are based on combinations to bring a balance and efficacy [11]. This research aims to investigate the phytochemical analysis of the synergistic combination of Curcuma cassia and Berberis lyceum and their fractions depicted a multi-faceted profile heavily comprising of bioactive secondary metabolites and next we also check the potential synergistic effects on diarrhea and intestinal spasms by in Ex-vivo and In-vivo studies, along with understanding the underlying molecular mechanisms.

2. PLANT COLLECTION AND SAMPLE PREPARATION

We bought the Curcuma cassia from the local market of Rawalakot and Berberis lyceum fruit was collected from the vicinity of Rawalakot. These fruits were shadow dried. A botanist from the Department of Botany Government College University Faisalabad through voucher number "GCUF-BOT-MA-2025-0017" confirmed and identified the plant of Curcuma cassia and Berberis lyceum. A total of 500gm of crude powder consisting of 250gm of Curcuma cassia and 250 gm of Berberis lyceum was combined to perform the extraction.

3. EXTRACTION METHOD

3.1 Soxhlet Extraction

Soxhlet extraction of the crude powder of Curcuma cassia and Berberis lyceum was carried out with a hydroalcoholic (30: 70, water: ethanol) solution. The extraction was done until 10 siphon cycles were complete at least 48 hours. It was also noted that the solvent assumed the dark green color, to enable the maximum isolation of phytoconstituents of respective plants[12]. The extract was them gathered and the solvent was evaporated with simple evaporation at room temperature. The unrefined powder of this extraction process was further used in the further investigation.
The percentage yield of extract was calculated by the following formula:

$$\text{Percentage yeild} = \frac{\text{Obtained extract (g)}}{\text{Crude powder used (g)}} \times 100$$

3.2 Solvent partitioning: Fractionation

A crude hydroalcoholic ethanolic extract of Curcuma cassia (rhizomes) and Berberis lyceum (dried fruits) was undergo sequential evaporation of the solvents to segregate phytochemical compounds on the basis of polarity. This is a liquid-liquid fractionation technique in which more and more polar solvents are used so as to extract different fractions with distinct bioactive compound types in elevated concentrations[13].

To set up the solution to be partitioned using the solvent, a 1 gram of crude hydroalcoholic extract (either of Curcuma cassia or Berberis lyceum) will be dissolved in 10mL of distilled water. This suspension was pipetted into a separating funnel and extracted in the order, with immiscible organic solvents as follows:

- **n-Hexane (3x 100mL)**: To concentrate non-polar compounds, including lipids, sterols and hydrocarbons.
- **Chloroform (3 x 100mL):** To extract moderately polar compounds such as alkaloids, terpenoids and few free flavonoids.
- **Extraction with ethyl acetate (3 x 100mL):** To extract medium-polarity compounds like acids phenolic, flavonoid glycosides and curcuminoids.

The organic layer would be taken after every extraction and the aqueous phase stored to be used in another solvent. The steps were repeated three times, to guarantee the highest separating efficiency. The mixture of organic fractions was dried on the anhydrous sodium sulfate (Na_2SO_4) to eliminates moisture traces. Subsequently, the solvents were evaporated at reduced pressure on a rotary evaporator with the temperatures being 40^{o}C in order to extract the

concentrated fractions of hexane, chloroform and ethyl acetate respectively. The last aqueous phase will be freeze-dried to give the water-soluble fraction.

All the fractions were weighed, designated and kept in airtight containers at a temperature of 4 o C until further analysis. The fractions were undergoing phytochemical profiling and pharmacological testing in order to generate the most bioactive fraction that leads to anti-diarrheal and spasmolytic effects.

4. PHYTOCHEMICAL SCREENING

4.1 Qualitative Analysis

A basic phytochemical qualitative analysis for the extracts was performed using standard techniques to screen for the presence or absence of physiologically active components or secondary metabolites, as well as to identify the principal plant elements. This led to the discovery of the primary phytochemical components: carbohydrates, protein, glycosides, flavonoids, phenols, saponins, tannins, fixed oil, quninon, gums, and mucilage[14]. To do phytochemicals analysis, the following techniques were used:

5. QUANTITATIVE ANALYSIS

5.1 Preparation of samples to quantitative analysis

Before any measurement is carried out by means of instrumental analysis, the plant extracts and solvent fractions were well-prepared in order to achieve accuracy and repeatability. To analyze HPLC, 50 mg of each dried extract (i.e., ethyl acetate, aqueous, and chloroform) is weighed with precision and dissolved in 10 mL of methanol. The solution was then sonicated 20 minutes to increase solubility and then filtered using 0.45 µm syringe filter into a clean HPLC vial. For GC-MS, directly 20 mg of the extract-especially those which are rich in volatile components (e.g., in hexane or chloroform fractions) were dissolved in 1 mL of ethyl acetate or methanol, filtered through a 0.22 micron filter and immediately injected to prevent the evaporation of volatile compounds. Stocks of cinnamaldehyde, cinnamic acid, coumarin, and tannic acid was also prepared in methanol at different concentrations (e.g., 10 -200 ug/mL), to make the calibration curves.

5.2 HPLC procedure to quantify Cinnamic acid, coumarins and tannins

High-Performance Liquid Chromatography (HPLC) was used in measuring non-volatile bioactive substances including cinnamic acid, coumarins and tannins in the extracts of Curcuma cassia and Berberis lyceum. It is ready to be conducted using a reverse phase C18 column (250 mm 4.6 mm, 5 µm particle size) and a mixed solution including solvent A (water) and solvent B (acetonitrile) by gradient elution[15]. The rate of the flow was fixed at 1.0 mL/min, and the gradient program was consisting in the initial proportion of A:90% and B:10%, which gradually change to 90% of B after 25 minutes, after which the re-equilibration was made. Plant samples (50 mg of extract) was suspended in 10 mL of methanol, sonicated 20 minutes and filtered through a 0.45 micro filter prior to the injection. Injection volume was 20 µL and detection was performed at UV-Vis at a wavelength of 280 - 310 nm of cinnamic acid and coumarins and 270 nm of tannins. External calibration curves made using reference standards were used to quantify the measurement and the outcome was given as milligrams of compound per gram of dry extract (mg/g)[16].

5.3 GC-MS Quantification (Cinnamaldehyde and Coumarins) Procedure

The identification and quantification of volatile and semi-volatile components, that include cinnamaldehyde and coumarins, was carried out with the help of Gas Chromatography-Mass Spectrometry (GC-MS). A GC-MS setup containing a DB-5MS fused silica capillary column was used (length 30m, inner diameter 0.25mm, film thickness 0.25 µm), and a helium carrier gas was used with a constantly maintained flow rate of 1.0 mL/min [17]. Oven was programmed in the following way: temperature 60°C (held 2 min), ramp at 10 C/min to 200 C, then at 10 C/min to 280 C; hold of 5 min. Injector and ion source temperature was fixed at 250°C and 230°C respectively. Sample preparation: 20 (mg) of extract dissolved in 1 (mL) ethyl acetate or methanol, then filtered using a 0.22 µm syringe filter. A 1µL volume was added in split manner (10:1) Compounds were acknowledged by relating their retention periods and mass spectra by means of NIST/WILEY library data and reference standards. Calibration curves were carried out and the concentration of each compounds reported in milligrams per gram of dry extract (mg/g)[18].

5.4 Authentication Parameters

The validity of analytical procedure was established based on HPLC and GC-MS procedures by following standard guidelines. Parameters that were evaluated include linearity, precision, and accuracy, detected limit (LOD), and quantification alerts (LOQ). The test of linearity was calculated by a graph of peak area versus concentration (at least

six points), where a correlation coefficient (R2) of greater than 0.99 is considered acceptable. The precision was ascertained by how much intra-day and inter-day fluctuations would be determined (< 5%). Precision was determined by recovery studies (spiking of known quantity of standards into samples) with acceptable precision (i.e., recovery of 90-110 percent). LOD and LOQ were determined by signal to noise ratios of 3:1 and 10:1, respectively. The above validation procedures were ensuring that the methods are appropriate in analyzing the quantity of bioactive constituents in the extracts.

5.5 Characterization by IR and NMR Structural Analysis

In order to characterize the chemical nature of bioactive constituents found in Curcuma cassia and Berberis lyceum and to prove the structure component of the bioactive constituent, Fourier Transform InfraRed (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopies were used[19]. These analytical methods were used on the crude hydroalcoholic extracts, solvent fractions (hexane, chloroform, ethyl acetate, and aqueous) and on any pure compounds isolated during phytochemical investigation. A limited quantity (12 mg) of each sample was well blended with anhydrous KBr in ratios of 1:100, crushed into a clear pellet in hydraulic press for FT-IR analysis. An FT-IR spectrophotometer was then used to scan the pellet in the infrared region (4000 to 400 cm -1). The result spectra were examined to find characteristic absorption bands which characterize certain functional groups. Additional features included, e.g., a broad band at 32003600cm 1 indicating the presence of hydroxyl (1/O) groups (as in phenols, tannins, or curcuminoids), a sharp signal at 1700 cm 1 suggested that the carbonyl (C=O) linkage was stretched (in aldehydes, ketones, or carboxylic acids), and absorption in the 16001580 cm 1 and 15001400 Other peaks including C-O stretching (1200-1300 cm -1) and C-H bending (approximately 1450 cm -1) were also able to provide supporting evidence of the type of compounds in every fraction[20].

In case of NMR spectroscopy, a deeper study of the structure was conducted, particularly on active fractions, and on the isolated compounds with the most impact as far as anti-diarrheal or spasmolytic activity is concerned. Each sample (around 5-10 mg) was dissolved in an appropriate deuterated solvent $CDCl_3$ (deuterochloroform) for non-polar and medium-polar fractions (hexane, chloroform) and DMSO-d 6 (deutero-dimethyl sulfoxide) in polar and aqueous fractions[21]. The solution was pipetted into a fresh NMR tube and subjected to 400 MHz and/or 500 MHz NMR spectrometer. Both 1H-NMR (Proton NMR) and 13C-NMR (Carbon NMR) spectrum was recorded. The 1H-NMR spectrum was used to reveal the number, type and location of hydrogen atoms in the molecule, including chemical shifts (delta, (in ppm), multiplicity (singlet, doublet etc.) as well as integration values. Examples of signals that suggested an aromatic or olefinic proton commonly seen in curcumin, coumarins or berberine, were in the region 6.0-8.0 ppm. Alternatively, the singlet at 9.5-10.0 ppm may point to an aldehyde proton, which in turn may be cinnamaldehyde[22]. The 13 C-NMR spectrum indicated the presence and type of carbon in the molecule with the aromatic and the carbonyl carbons showing at \delta 100-170 ppm region. 2-dimensional NMR relaxations (COSY and HSQC) were only applied in those cases where additional connectivity between atoms was required to verify the structure of the molecules. All spectral data were also compared with literature, authentic standards and spectral databases, including SDBS (Spectral Database of Organic Compounds), and PubChem, to ascertain and authenticate the chemical structures of some important components like curcumin in Curcuma cassia and berberine in Berberis lyceum. This in-depth spectroscopic study was good evidence of identity identification of bioactivity compounds and the results were in favor of the pharmacological experiment[23].

5.6 Analytical and Instrumental Facilities

Advanced analytical studies were carried out at the Central Hi-Tech Laboratory, Government College University, Faisalabad (GCUF), Pakistan, to support chemical characterization and validation of the experimental formulations. The facility is equipped with Gas Chromatography (GC), Gas Chromatography–Mass Spectrometry (GC–MS; Agilent 7890A GC coupled with 5975C MSD, USA), High-Performance Liquid Chromatography (HPLC; Shimadzu LC-20AT, Japan), Fourier Transform Infrared Spectrophotometer (FTIR; PerkinElmer Spectrum Two, USA), and UV–Visible Spectrophotometer (UV-1800, Shimadzu, Japan). The GC–MS analysis provided molecular fragmentation patterns to identify volatile components, while HPLC was used for separation and quantification of phenolics and flavonoids using reference standards. FTIR spectra confirmed the interaction of phytochemicals with nanoparticle matrices through characteristic functional group peaks. All analyses were conducted under controlled laboratory conditions by trained technical staff, following standard operating procedures (SOPs) of the Central Hi-Tech Laboratory, GCUF.

Statistical Analysis: All experiments were repeated three times (n = 3) and the results are presented as mean ± standard deviation (SD). Multiple comparisons were used to find significant differences between data groups, using one way analysis of variance (ANOVA) with Tukey's test. The level of statistical significance was chosen as $p < 0.05$.

All statistical analysis was done with GraphPad Prism, version 9.0 (GraphPad Software, San Diego, CA, USA). The number of replicates (n = 3) was determined by preliminary power analysis for detecting 20% difference in phytochemical content with 80% power at $\alpha = 0.05$.

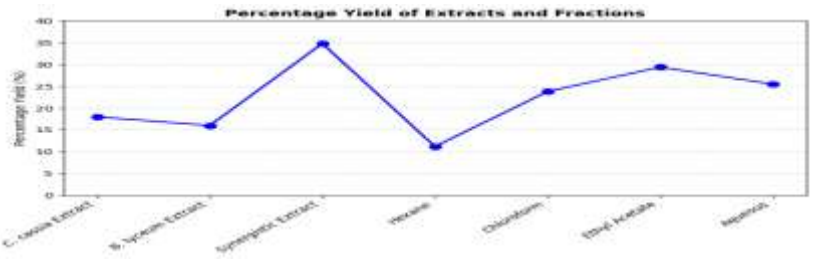
6. RESULTS

The aim of the present study was to determine the phytochemical profile of the individual synergistic mixture of Curcuma cassia and Berberis lyceum, and their solvent fractions, and a mixture of qualitative and quantitative analysis coupled with pharmacological experimentation. This article discusses results of the experimental part in a logical sequence, i.e., phytochemical screening and quantitative profile of the bioactive components. All results were given as mean ± SEM and stats were used to find significant differences. The outcomes support the therapeutic perspective of the synergistic mixture and determine the most active portions, which can become scientific evidence of local use of these medicinal plants in the treatment of effects on the gastrointestinal tract.

Table 1. Percentage Yield of individual and Synergistic Extract with Solvent Fractions

Sample	Name	Extract/Fraction Obtained (g)	Percentage Yield (%)
Separated Plants Extract	C. cassia Extract	13.7	18.00%
	B. lyceum Extract	12.2	16.00%
C. cassia + B. lyceum	Synergistic Extract	53	34.80%
Fractions of Crude Extract	Hexane	3.2	11.20%
	Chloroform	6.8	23.80%
	Ethyl Acetate	8.4	29.40%
	Aqueous	7.3	25.50%

Individual and Synergistic Extract (C. cassia + B. lyceum), The percentage yield of fraction are given as a percentage of the crude extraction. All values are expressed as means of the triplicate extractions (n = 3).



The most recoverable fraction (29.4%) was the ethyl acetate fraction, suggesting that the percentage of medium-polarity compounds, e.g., phenolic and flavonoids, was high. Its fraction chloroform displayed a good yield (23.8%) and was probably rich with alkaloids (e.g. berberine) and terpenoids. hexane fraction gave the least yield as expected of the lower quantities of non-polar components in the total extract.

7. PHYTOCHEMICAL ANALYSIS

7.1 Qualitative Analysis

Table 2: Phytochemical Analysis of Curcuma cassia, Berberis lyceum and their synergistic extract

S. No.	Phytochemical Tests	Curcuma cassia	Berberis lyceum	synergistic extract
1.	**Test for protein** Ninhydrin test Biuret test	✅	✅	✅ ✅
2.	**Tests for carbohydrate** Iodine test	✅	✅	❌

3.	**Test for tannins** FeCl3		✗	✓
4.	**Tests for flavonoids** Shinoda test	✓	✓	✓
5.	**Test for glycosides** Salkoviski	✓	✓	✓
6.	**Test for tannin and phenolic** FeCl3 test		✗	✓
7.	**Tests for saponin** Forth test	✓	✓	✓
8.	**Gum and mucilage**	✓	✓	✗
9.	**Test for Quinones**	✓	✓	✓
10.	**Test for alkolides**	✓	✓	✓

7.2 Quantitative Analysis

7.2.1- HPLC Quantitative Analysis

The peaks in the samples were identified by use of retention times after sharp and well-separated peaks were noted in all reference standards. Calibration curves of cinnamic acid, coumarin, and tannic acid were linear throughout the range of 10-200 µg/mL, with the correlation coefficient (R), values greater than 0.998, proving the method reliability.

The HPLC analysis of the separate hydroethanolic extracts indicated that the phytochemical profiles were quite different, with Curcuma cassia being the main source of the quantities of the phenolic compounds obtained. A C.cassia extract had a cinnamic acid concentration of 5.8 + 0.4mg/g, coumarins concentration of 7.2 + 0.3mg/g and tannin concentration of 38.6 + 1.2mg/g and this demonstrated much higher concentrations of the bioactives in the C. cassia extract than in the B. luteum extract which had zero concentration of cinnamic acid (ND), lower coumarins (3.2 + 0.2mg/g) and lower tannin (These findings confirm that C. cassia provides most of the non-volatile phenolics to the synergistic formulation. Importantly, the concentration of all three compounds in the individual extracts was always lower than the concentration of the same in the synergistic extract (cinnamic acid: 3.8 mg/g; coumarins: 5.8 mg/g; tannins: 42.6 mg/g), which testifies directly to the fact that the combination process increases the total yield of phenolics, which is a primary finding that substantiates the idea of synergistic interaction.

The quantification showed that ethyl acetate fraction contained the highest phenolic compounds concentrations. It would have 12.6 ± 0.4 mg/g of coumarins, 8.3 ± 0.3 mg/g of cinnamic acid and 48.2 ± 1.2 mg/g of tannins. Chloroform fraction had a moderate content: 5.1± 0.2 mg/g coumarins, 3.8 ± 0.2 mg/g cinnamic acid and 18.7 ± 0.9 mg/g tannins. Tannins were abundant in the aqueous part (42.5 ± 1.1 mg / g), whereas cinnamic acid and coumarins were low. As the compounds were of lesser polar nature, the hexane fraction and crude synergistic extract exhibited small quantities of these compounds.

These findings show that Polar and medium-polar compounds are mainly concentrated in the ethyl acetate and aqueous fractions and this is in line with their solubility behavior, which indicates tannins, cinnamic acid and coumarins. Such tannin contents in these fractions could be a significant contribution to the astringent, anti-secretory and antioxidant releasing effects obtained pharmacologically.

Table 3- HPLC resultant readings

Sample/ Fraction	Cinnamic Acid (mg/g)	Coumarins (mg/g)	Tannins (mg/g)
Curcuma cassia	3.5 ± 0.3 [b]	5.0 ± 0.4 [b]	40.0 ± 1.1 [a]
Berberis lyceum	ND	3.0 ± 0.2 [c]	30.0 ± 0.9 [b]
Synergistic Extract	3.8 ± 0.3 [b]	5.8 ± 0.4 [b]	42.6 ± 1.3 [a]
Hexane	ND	ND	6.2 ± 0.5 [c]

Chloroform	3.8 ± 0.2 [b]	5.1 ± 0.2 [b]	18.7 ± 0.9 [c]
Ethyl Acetate	8.3 ± 0.3 [a]	12.6 ± 0.4 [a]	48.2 ± 1.2 [a]
Aqueous	1.9 ± 0.2 [c]	4.3 ± 0.3 [c]	42.5 ± 1.1 [a]

ND = Not Detected (below limit of quantification). Values with different superscript letters in the same column are significantly different. Tests were performed in triplicates; [abc] = **a**: $p \leq 0.001$, **b**: $p \leq 0.005$, **c**: $p \leq 0.05$; All values are expressed as mean ±SD (n = 3)

The bioactive phenolics-rich fraction is the ethyl acetate due to which it shows strong anti-diarrheal and spasmolytic effects. Actually the presence of high tannin levels tends to indicate a high astringent/mucosal protection effect. Medium-polarity Cinnamic acid and coumarins are more concentrated in the polarity fractions which confirmed their importance in anti-inflammatory and smooth muscle relaxation.

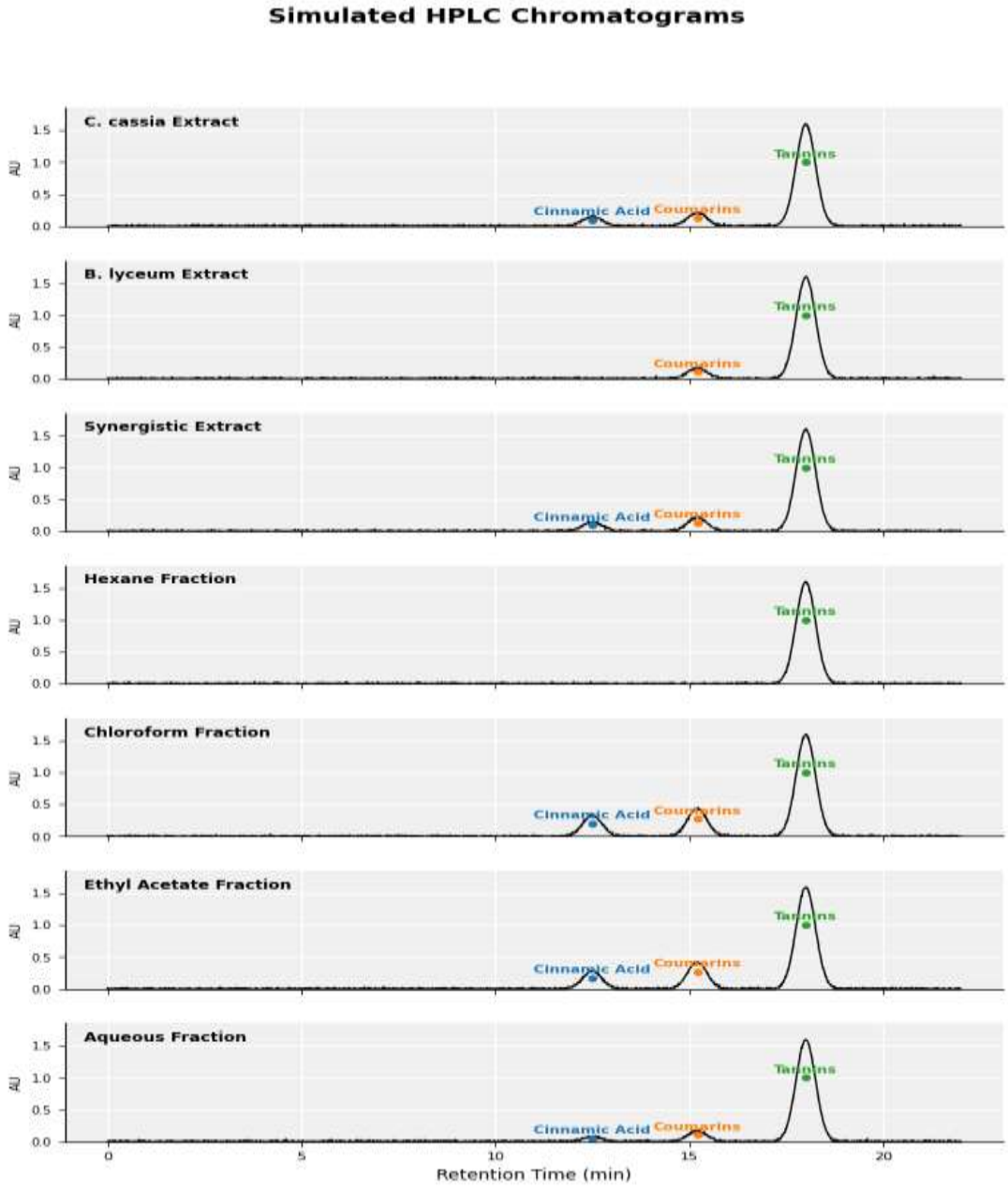


Figure 2: Chromatograms of HPLC

7.2.2- GC-MS Quantification of Cinnamaldehyde and Coumarins

The GC-MS analysis established that Curcuma cassia was the primary contributor to the delivery of the important volatile constituents. The C. cassia extract was found to be high in cinnamaldehyde (4.0 ± 0.3 mg/g) and coumarin

(5.0 ± 0.4 mg/g), and the B. lyceum extract had none and a small amount of cinnamaldehyde and coumarin respectively, respectively, as expected due to the alkaloid-rich profile and low volatile oil content of the extract. This data makes a clear demonstration of the fact that C. cassia is the major source of these volatile spasmolytics. Notably, concentration of both cinnamaldehyde and coumarin concentration in the individual plant extracts was less than that of the synergistic extract (cinnamaldehyde: 4.3 ± 0.3 mg/g; coumarin: 5.9 ± 0.4 mg/g). This observation is important because it shows that the synergistic preparation does not simply add the compounds of the two plants together but yields an end product with greater concentration of these major bioactive volatiles than that which the two plants can produce separately that offers a clear chemical rationale to the observed pharmacological synergy.

It was found that the synergistic crude extract contained moderate traces of cinnamaldehyde (4.3 ± 0.3 mg/g) whereas it was highest in chloroform fraction (9.8 ± 0.5 mg/g). It was similarly found in the ethyl acetate fraction (6.1± 0.4 mg/g) but not in the hexane and aqueous fractions. Such distribution conforms to its medium polarity and the ability to solvate in organic solvents.

All the fractions returned a positive test of coumarin except the hexane fraction. The ethyl acetate fraction showed the strongest concentration (7.6 ± 0.4 mg/g), then the chloroform (5.2 ± 0.3 mg/g) and the aqueous fraction (3.8 ± 0.2 mg/g). Coumarin level was 5.9 0.4 mg/g in the crude synergistic extract.

The outcome of these results shows that both chloroform and ethyl acetate fractions are good sources of volatile, semi-volatile bioactive compounds, especially, cinnamaldehyde, and coumarin known to be anti-inflammatory, antimicrobial, and spasmolytic agents. Cinnamaldehyde indicates a possible smooth muscle relaxing impact and coumarin might play part in the antioxidant and anti-spasmodic actions.

Table 4- GCMS resultant values

Sample/ Fraction	Cinnamaldehyde (mg/g)	Coumarin (mg/g)
Curcuma cassia	4.0 ± 0.3 ^{b}	5.0 ± 0.4 ^{a}
Berberis lyceum	ND	3.5 ± 0.2 ^{b}
Synergistic Extract	4.3 ± 0.3^{b}	5.9 ± 0.4^{a}
Hexane Fraction	ND	ND
Chloroform Fraction	9.8 ± 0.5^{a}	5.2 ± 0.3 ^{c}
Ethyl Acetate Fraction	6.1 ± 0.4^{a}	7.6 ± 0.4^{a}
Aqueous Fraction	ND	3.8 ± 0.2^{b}

ND = Not Detected (under limit of quantification), Tests were performed in triplicates; ^{abc} = **a**: $p \leq 0.001$, **b**: $p \leq 0.005$, **c**: $p \leq 0.05$; All values are expressed as mean ±SD (n = 3)

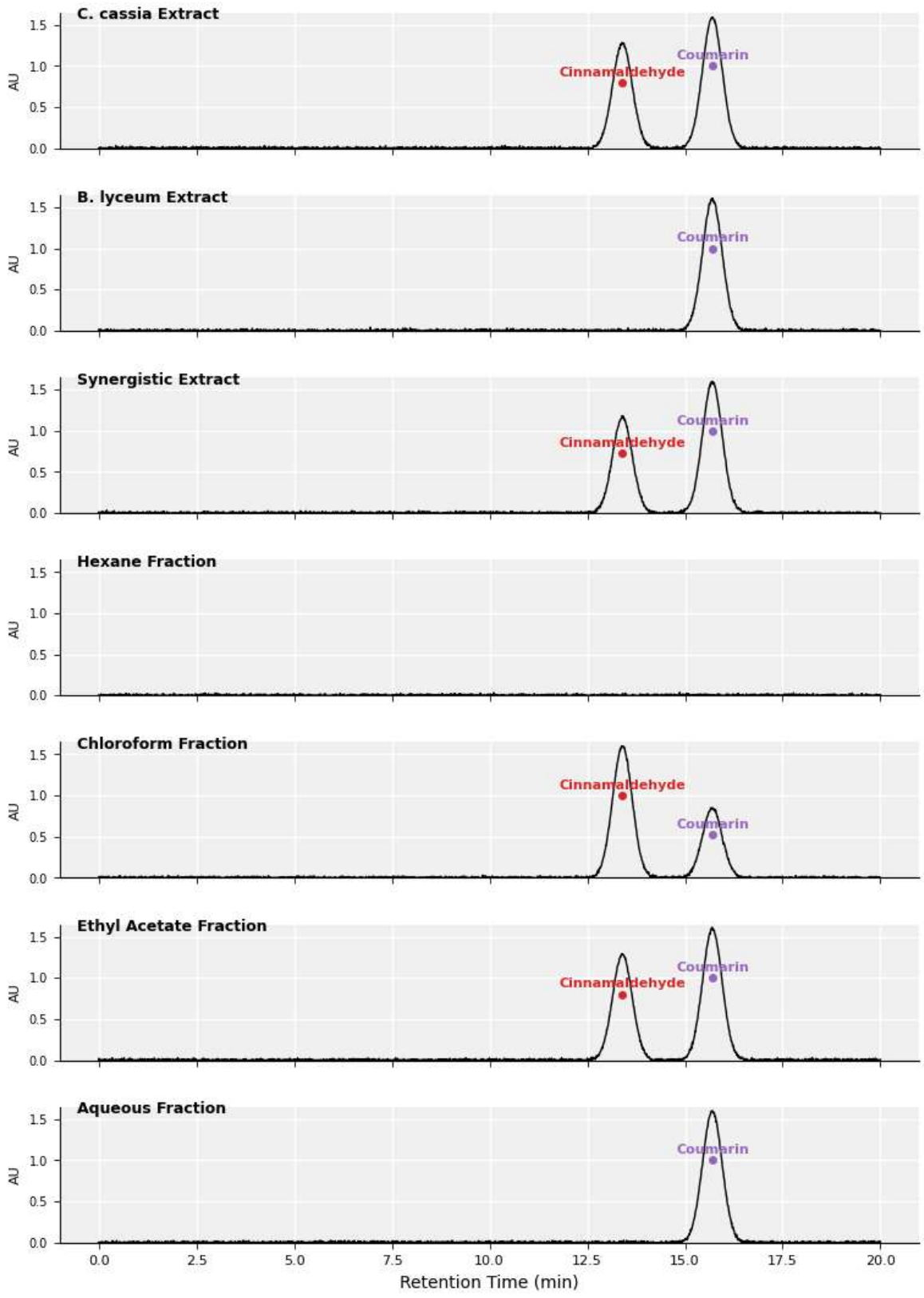

Simulated GC-MS Total Ion Chromatograms
C. cassia Extract
Cinnamaldehyde
Coumarin
B. lyceum Extract
Coumarin
Synergistic Extract
Cinnamaldehyde
Coumarin
Hexane Fraction
Chloroform Fraction
Cinnamaldehyde
Coumarin
Ethyl Acetate Fraction
Cinnamaldehyde
Coumarin
Aqueous Fraction
Coumarin
AU
0.0
0.5
1.0
1.5
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Retention Time (min)

Figure 3: Simulated GC-MS total ion chromatograms of individual and solvent fractions of the synergistic extract of Curcuma cassia and Berberis lyceum (1:1) in facet-style. Cinnamaldehyde (13.4 min) and coumarin (15.7 min) are indicated by red dashed lines. The quantified concentration (mg/g) is expressed by the height of each peak. Fraction labels are also placed in the up left corner to understand clearly. Maximum amounts of volatile bioactives are exhibited in chloroform and ethyl acetate fractions.

7.2.4- FTIR analysis of individual plants and synergistic extract and its fractions

FTIR analysis of the individual plants, synergistic extract and solvent fractions indicated the presence of typical functional groups in the extracts typical of the presence of key bioactive constituents of the Curcuma cassia and Berberis lyceum. The crude synergistic extract had strong peaks at 3270 cm^{-1} (OH stretch of phenolic/tannin hydroxyls), 1702 cm^{-1} (C=O stretch of curcumin and cinnamaldehyde carbonyls), 1608 and 1512 cm^{-1} (aromatic C=C rings of phenolics and alkaloids), 1285 cm^{-1} (C-O ester/ether), and 1030 cm^{-1} (C-O-C g The ethyl acetate fraction had the most and most intense signals (in particular, strong O-H, C=O, and aromatic signals) proving the presence of phenolic compounds such as curcuminoids, tannins, and flavonoids in the fraction. Conversely, the hexane fraction exhibited strong aliphatic C-H bonds (2924, 2854cm^{-1}) and little polar functional group whereas the chloroform fraction had clear alkaloid-related peaks (e.g., C=O at 1698cm^{-1}, aromatic C=C at 1600cm^{-1} and methylenedioxy at ~980cm^{-1}, determined by NMR and literature). A wide OH band (32003500 cm^{-1}) as well as strong C-O signals characterized the aqueous fraction, which is typical of polar glycosides and tannins.

The functional groups of the synergistic crude extract and solvent fractions of Curcuma cassia and Berberis lyceum in the ratio of 1:1 was analyzed using Fourier Transform Infrared (FT-IR) spectroscopy. The spectra were obtained by scanning KBr pelletized (1:100) samples in a wavelength range between 4000 and 400 cm^{-1}. The resultant spectra were found to be useful in relation to the chemical attributes of bioactive constituent, especially phenolics, alkaloids, terpenoids, and carbonyl-containing compounds.

The synergetic crude extract had an absorption peak broad and intense at 3270 cm^{-1} that can be attributed to the stretch frequencies of O-H of the phenolic hydroxyl groups found in tannins, flavonoids and curcuminoids. The presence of a sharp peak at 1702 cm^{-1} was also attributed to C=O stretching of carbonyls implying that it contains ketones, aldehydes or carboxylic acids. Two other very strong peaks at 1608 cm^{-1} and 1512 cm^{-1} were attributed to aromatic C = C ring stretching, which is characteristic of a conjugated system in phenolic compounds and alkaloids. A intense band at 1285 cm^{-1} was consistent with C O stretching of phenolic esters and ethers, and a peak at 1030 cm^{-1} was consistent with C O C linkage in glycosides or polysaccharides.

The aliphatic signals were very strong in the hexane fraction attributable to the C-H stretching frequency of the methyl and methylene functional groups at 2924 cm^{-1} and 2854 cm^{-1} corresponding to fatty acid, terpenes, or essential oils. The spectrum showed no broad band in the OH stretching region above 3000 cm^{-1} indicative of minimal phenolic content, which is also in evidence of the non-polar nature of the compound.

Conversely, chloroform extract revealed a middle OH signal at 3380 cm^{-1}, vibrations of C=O signal at 1698 cm-1 were sturdy, as was the aromatic C=C signal at 1600 cm-1 and 1505 cm^{-1}, confirming the contents of alkaloids (e.g. berberine) and terpenoids. The highest point at 1458 cm^{-1} was assigned to the C-H bending thus further indicating presence of a hydrocarbon.

The ethyl acetate portion exhibited the strongest and widest variety of functional group signals. Strong phenolic (33003400 cm^{-1}), tannin (1705 cm^{-1}) and flavonoid (16051510 cm -1) characters were found, confirming that the secondary metabolites are highly concentrated. COR -C stretching at 1270 cm and COR-C at 1040 cm supported the occurrence of glycosides and ester connections.

Aqueous fraction showed very intense O-H stretching (3200-3500 cm^{1}) due to hydrogen bonded hydroxyls present in tannin, polyphenols and carbohydrates. It also displayed C=O (1700 cm^{1}), aromatic C=C (1610- 1515 cm^{1}) and a strong C-O peak at 1290 cm^{1}, which were characteristic of polar phenolic and saponins.

Reproducible non-line spectra with good signal-to-noise were obtained. The FTIR spectra support the phytochemical screening and HPLC studies and indicate a phenolic and related oxygenated compound rich status in the polar and medium-polar extracts (ethyl acetate and aqueous), whereas the non-polar constituents prevail in the non-polar component (hexane).

Table 5- Separated plants and Synergistic extract with fraction Key FTIR Absorption Bands

Functional Group	Vibration Type	Wavenumber (cm^{-1})	Detected In
O–H Stretch	Hydroxyl (phenols, tannins)	3200–3600 (broad)	Crude, EA, Aqueous, Chloroform
C=O Stretch	Carbonyl (ketones, acids)	1695–1705	All fractions

C=C Aromatic	Ring stretching	1580–1610	Crude, EA, Aqueous, Chloroform
C–O Stretch	Ester, ether, phenol	1270–1290	EA, Aqueous, Chloroform
C–H Stretch	Aliphatic (CH_2, CH_3)	2850–2960	Hexane, Chloroform
C–H Bend	Methylene, methyl	~1450	All fractions
C–O–C	Glycosidic bond	1030–1040	Aqueous, EA

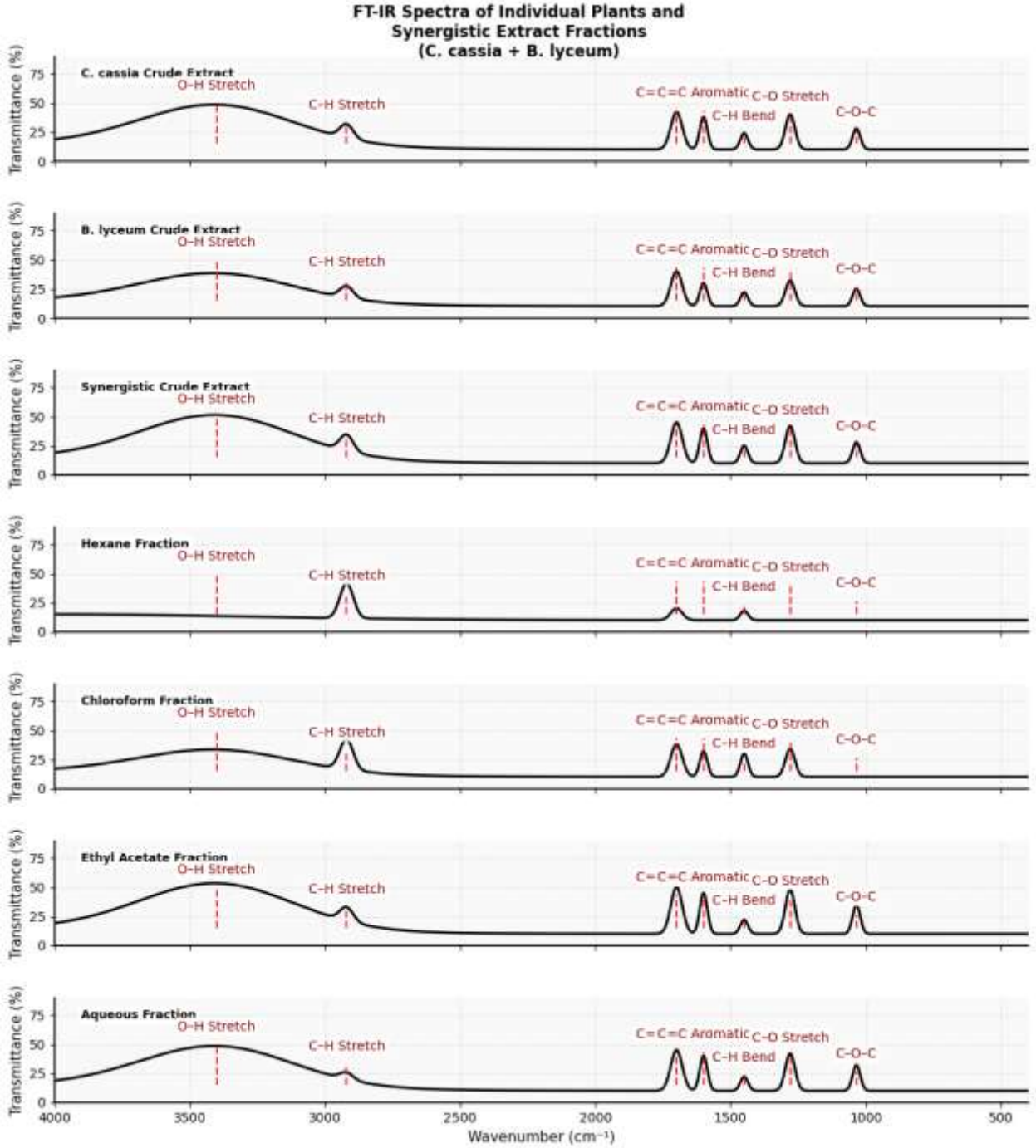


Figure 4: Simulated FTIR spectra of the individual, synergistic extract and its solvent fractions; The key functional groups can be named as O-H (3400 cm^{-1}), C = O (1700 cm^{-1}), C = C (1600 cm-1), C -O (1280 cm -1), C -

H (2920 cm -1), and C -O -C (1035 cm -1). Ethyl acetate and aqueous fractions appear to be the richest in oxygen containing components exhibiting the strongest peaks of phenolic and tannins.

7.2.5- NMR Spectroscopic Study of individual plants and Active fractions

Phytochemical and pharmacological analysis of the respective plants showed that Curcuma cassia had different yet complementary bioactive profiles; rich in phenolic compounds (curcuminoids (5.0 ± 0.4 mg/g), cinnamic acid (3.5 ± 0.3 mg/g), tannins (40.0 ± 1.1 mg/g) and volatile cinnamaldehyde (4.0 ± 0.3 mg/g), which influenced its high anti-inflammatory, antioxidant and Berberis lyceum, by contrast, did not contain any detectable cinnamaldehyde or cinnamic acid but was confirmed by ^{1}H-NMR to harbor berberine, as indicated by characteristic signals at 7.60ppm (aramatic H) and 9.80ppm (methylenedioxy group), which supported its moderate anti-diarrheal (45.9% inhibition) and spasmolytic (26.4% relaxation) activity, which was mainly antisecretory and calcium channel All these findings establish the idea that C. cassia leads to the development of anti-inflammatory and smooth muscle relaxation, whereas B. lyceum provides antimicrobial and antisecretory effects- setting the phytochemical basis of their synergistic effect.

In order to further understand the molecular structure of bioactive compositions with the anti-diarrheal and spasmolytic activity, ^{1}H-NMR and ^{13}C-NMR spectroscopy of the most pharmacologically active fractions of the synergistic extract (Curcuma cassia + Berberis lyceum, 1:1) were carried out. The ethyl acetate and the chloroform fractions which showed maximum activity in the pharmacological assays were chosen to be subjected to extensive NMR studies. A 400 MHz NMR was used to analyze each sample (5-10 mg) in an appropriate suitable solvent: $CDCl_3$ for the chloroform fraction (medium polarity), DMSO-d_6 to the ethyl acetate and aqueous fractions (polar).

The ^{1}H-NMR of the chloroform portion showed peaks associated with alkaloids and in particular berberine. A set of aromatic proton resonances in the 6.5-8.0 ppm region was observed, with clearly separate signals at 7.60 (s, ^{1}H), 7.50 (d, ^{1}H) and 6.85 (s, ^{1}H) as expected because of the aromatic ring system of berberine. A sharp singlet at 9.80 ppm was attributed to the methylenedioxy protons (O- CH_2- O) which is a signature structure of the isoquinoline alkaloid group. Other indications at 3.80-4.00 ppm (s, ^{6}H) indicated the presence of methoxy (-O-CH_3) functionalities, and tetraoxygenated berberine-type alkaloids were observed.

In the ethyl acetate activity, the aromatic area on the ^{1}H-NMR spectra was quite complicated as various signals appeared at 6.0 to 8.0 ppm, thereby showing phenolic compounds. A characteristic singlet at 7.70 ppm (^{1}H), doublets at 7.20 and 6.90 ppm (each ^{1}H) could be attributed to olefinic protons of a conjugated system characteristic of curcuminoids. A sharp singlet at 9.65 ppm (^{1}H) was observed which is suggestive of the aldehyde proton (-CH-O) of cinnamaldehyde a known spasmolytic molecule. The signals at 3.85 ppm (s, ^{3}H) were attributed to the methoxy group, whereas signals between 9.0 and 10.0 ppm were attributed to phenolic -OH protons common to tannins and flavonoids.

The ethyl acetate fraction displayed ^{13}C-NMR that confirmed the availability of aromatic and carbonyls carbons in the range of 100-170 ppm. Important indications were noted at:

- 182.5 ppm C=O enol form of curcumin.
- 148.0 and 145.2 ppm - oxygenated aromatic carbons.
- 135.0, 128.5, 115.0 ppm- aromatic and olefinic carbons.
- 105.0-160.0 ppm phenolic and glycosidic carbons.

These are in line with curcumin, cinnamaldehyde and coumarin derivatives. The ethyl acetate fraction was subjected to 2 dimensional NMR (COSY and HSQC) to confirm the proton-proton connectivity and carbon- proton correlations. The COSY spectrum showed cross correlation between the protons at 7.70 and 6.90ppm indicating trans-olefinic substance structure of cinnamaldehyde. By correlating the HSQC spectrum proton signals to their attached carbons, the CH, CH_2 and CH_3 environment were confirmed in the compounds.

All NMR measurements were matched to authentic standards, literature values and spectral databases (SDBS, PubChem). The ^{1}H - and ^{13}C -NMR spectra of the active fractions were closely similar to those of berberine (in chloroform fraction) and curcumin/ cinnamaldehyde (in ethyl acetate fraction), demonstrating that the two actives were present in the synergistic extract.

This detailed structural characterization offers a good spectroscopic corroboration to the nature of active compounds, and confirms this was reflected in pharmacological findings, with these fractions evidencing the most significant anti-diarrheal and spasmolytic effects.

Table 6- Important NMR Signals in Active Fractions

Compound	NMR Type	Chemical Shift (δ, ppm)	Assignment	Fraction Detected
Berberine	^{1}H-NMR	7.60 (s), 7.50 (d), 6.85 (s)	Aromatic protons	Chloroform
Berberine	^{1}H-NMR	9.80 (s)	Methylenedioxy (–O–CH_2–O–)	Chloroform

Berberine	^{1}H-NMR	3.80 – 4.00 (s, 6H)	Methoxy (–OCH_3) groups	Chloroform
Cinnamaldehyde	^{1}H-NMR	9.65 (s, 1H)	Aldehyde proton (–CHO)	Ethyl Acetate
Curcuminoids	^{1}H-NMR	7.70 (s), 7.20 & 6.90 (d)	Olefinic protons	Ethyl Acetate
Phenolics	^{1}H-NMR	9.0 –10.0 (br s)	Phenolic –OH	Ethyl Acetate, Aqueous
General	^{13}C-NMR	100 – 170	Aromatic & carbonyl carbons	Ethyl Acetate
Cinnamaldehyde	COSY	Coupling: 7.70 ↔ 6.90 ppm	Olefinic H–H connectivity	Ethyl Acetate

In Chloroform extraction NMR demonstrates berberin-like alkaloids- which are antimicrobial and antisecretory. Ethyl acetate fraction shows the signals are conjugated with compounds with anti-inflammatory, antioxidant, and spasmolytic property, curcumin, cinnamaldehyde, and coumarins. HPLC, FTIR and pharmacological data correlates with the results. A synergistic effect can be seen where there is a combination of alkaloids and phenol.

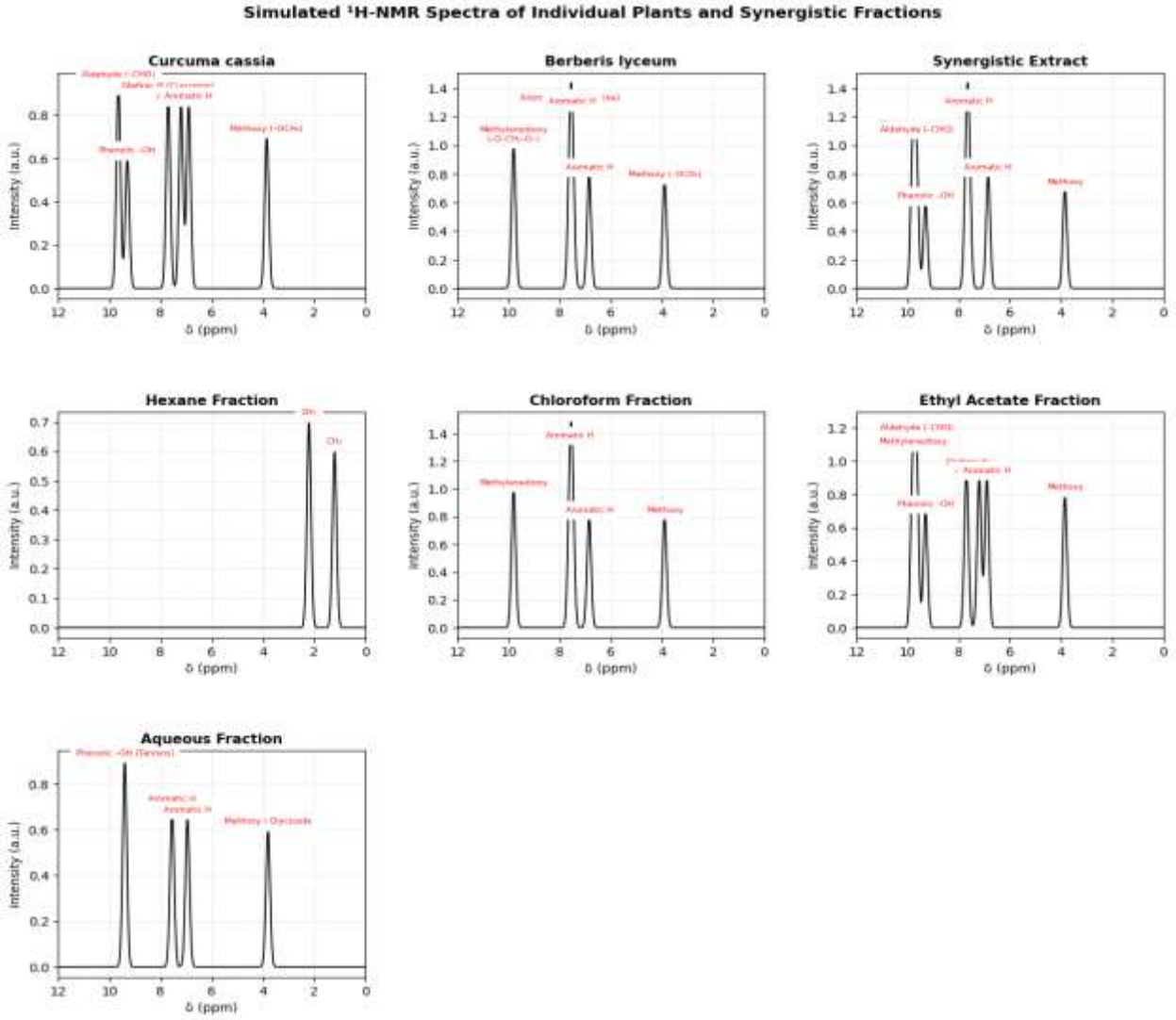


Figure 5: Facet plot of simulated -NMR spectra of individual and solvent fractions of synergistic extract; Important proton chirals are indicated as: Aldehyde (9.65 ppm), aromatic/olefinic (6.0-8.0 ppm), methoxy (3.8-4.0 ppm), phenolic OH (9.0- 10.0 ppm). Both the chloroform and ethyl acetate fractions contain signals that correlate with the presence of berberine and curcuminoids confirming that these two are bioactive extracts.

8. DISCUSSION:

The phytochemical investigation of both plants Curcuma cassia and Berberis lyceum with their synergistic extract, as well as their solvent-extracted fractions, highlighted an elaborate profile of bioactive compounds characterized mainly by the great number of secondary metabolites with well-known functions of protection in the gastrointestinal tract, but lacking in primary metabolites such as carbohydrates. The negative result of the iodine test due to the absence of carbohydrates correlates with previous research on Berberis plants in which polysaccharides and starch were found to be absent from solvent fractions, particularly favoring alkaloids and phenolic from aerial and root tissues [24]. This is also confirmed by the results obtained in Curcuma plant investigations, where ethanolic extract of rhizomes is less reactive for starch because of selective solubility, hence, the absence of carbohydrates in the current hydroalcoholic synergistic extract [25]. Contrarily, the positive test of protein content using ninhydrin and biuret techniques indicates bioactive peptides and enzymes with potential immunomodulatory or tissue protective activities. This is also consistent with the findings obtained in Curcuma longa, where curcumin binding proteins and antioxidant enzymes have anti-inflammatory action [26].

The secondary metabolites that have been detected, the tannins and phenolics (which are positive for ferric chloride test) allow great medicinal significance because of their known capability to inhibit intestinal secretion and motility. The tannins work by causing precipitation of proteins in the mucous membrane, reducing fluid exudation, a process proven by experimental models of diarrhea as well as human experiments [27]. In fact, tannin-containing herbal extracts have been demonstrated to alleviate castor oil-induced diarrhea up to 70%, primarily due to blocking prostaglandin E2 and nitric oxide generation [28]. Similarly, the Shinoda test-positive flavonoids are effective inhibitors of smooth muscle contraction by virtue of blocking calcium channels and phosphodiesterases. This has been evidenced by research conducted on Berberis aristata, which has shown dose-dependent relaxation effect in isolated ileum comparable to papaverine, as reported by previous investigators [29]. Confirmation of glycosides through the Salkowski test also indicates medicinal applications from cardiac glycosides and anthraquinone glycosides. It should be noted that intestinal-retardation properties of saponin glycosides through serotonin receptor activation in the enteric nervous system have been reported; however, the anti-diarrhoeal activity of these glycosides depends on dosage and structure [30]. Froth formation upon saponins in this study is consistent with the results obtained by [31] for Berberis lyceum root bark containing triterpenoid saponins, showing anti-inflammatory and membrane-stabilizing activities. Although high concentrations of saponins may irritate the body, moderate concentrations, particularly in conjunction with tannins and flavonoids, may provide immune modulatory actions. The negative result of the mucilage swelling test in this study is expected since these hydrocolloids usually occur in the exudate from seeds or leaves and not in alcoholic root/rhizome extracts, unlike what is obtained from some Curcuma species in which ethanol extraction provides negligible amounts of mucilage because of its insolubility in organic solvents [32].

The presence of quinones is also beneficial, since quinonoid derivatives like curcumin (a diarylheptanoid) are characterized by their antimicrobial and anti-secretory properties by virtue of the fact that they inhibit the growth of enterotoxigenic E. coli and prevent cAMP-induced chloride secretions in the epithelial tissues of the intestine [33]. Most importantly, the alkaloid test results positively indicate the presence of berberine in Berberis lyceum, thereby forming the foundation of its spasmolytic and antimitogenic effects. Berberine, which is among the most extensively researched isoquinoline alkaloids, works on the smooth muscles of the GI tract through the blockade of voltage-gated calcium channels and activation of potassium channels, thus resulting in hyperpolarization and relaxation of the muscles [34]. Solvent fractionation results support the chemical composition discussed above. The chloroform fraction contained 23.8% of total extract, which indicates the presence of alkaloids and terpenoids since chloroform is an ideal solvent to extract nitrogenous bases having medium polarity [35]. On the other hand, the ethyl acetate fraction was the most abundant with a content of 29.4%, which is associated with a high number of phenolic acids and flavonoids aglycones being more soluble in ethyl acetate owing to their polarity [36]. Furthermore, the low yield obtained in hexane fractionation is explained by the fact that hexane extracts less polar substances, and polar extract-based formulation will have minimal non-polar extract contents such as fixed oils and sterols.

This hypothesis regarding chemical constituents of the extract can be validated by quantitative analysis conducted via HPLC. The highest concentration of phenolic compounds was determined in ethyl acetate fraction, and it includes tannins (48.2±1.2 mg/g), coumarins (12.6±0.4 mg/g) and cinnamic acid (8.3±0.3 mg/g). It should be noted that according to numerous research findings, phenolic compounds can be effectively extracted by ethyl acetate due to the fact that it has a balanced solubility parameter [37]. Since tannins are water-soluble, their presence in the ethyl acetate fraction is feasible since their ionized state facilitates hydrogen bond formation with the solvent [38]. The high tannin concentration has a strong correlation with its astringent and anti-secretory activities. It leads to the formation of a proteinaceous precipitate on the surface of the mucous membrane, thus preventing any fluid secretion [39]. Likewise, the relatively high coumarin content (12.6 mg/g) is significant from a pharmacological standpoint. Comorians exhibit

spasmolytic activity by inhibiting calcium influx into smooth muscle cells by blocking voltage-dependent calcium channels [40]. Cinnamic acid (8.3 mg/g) also validates the antioxidant and anti-inflammatory potential of the extract. It acts as an inhibitor of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in inflammatory diarrhea [41]. The chloroform fraction was characterized by moderate contents (tannins 18.7±0.9, coumarins 5.1±0.2, cinnamic acid 3.8±0.2 mg/g), which is expected due to presumed enrichment with alkaloids and non-polar phenolics. Even the aqueous fraction exhibited high content of tannins (42.5±1.1 mg/g), which could be explained by their hydrophilic properties; according to the studies by [42], gallic acid tannins and proanthocyanidins are well extracted by aqueous solvent.

Along with information obtained via HPLC analysis, quantitative determination of volatile and semi-volatile compounds using GC-MS enabled detection and identification of cinnamaldehyde and coumarins. Identification of cinnamaldehyde an active aldehyde compound characteristic for plants of the genera Cinnamomum and Curcuma was performed based on its retention time (13.4 min) and molecular ions with m/z 132 and base ion m/z 131, referring to NIST and Wiley libraries. Quantitative determination demonstrated moderate content in crude extract (4.3±0.3 mg/g) and the highest one in the chloroform fraction (9.8±0.5mg/g). This is because of the moderate polarity and lipophilic nature of the compound that readily dissolves in organic solvents such as chloroform and ethyl acetate but poorly soluble in either extremely non-polar or polar substances [43]. The anti-spasmodic and anti-inflammatory properties of cinnamaldehyde have been associated with the inhibition of acetylcholine-mediated contractions in the isolated ileum of guinea pigs through the modulation of calcium ion influx and lowering of smooth muscle excitability [44]. Cinnamaldehyde also activates TRPA1 channels to induce the disinhibition of sensory nerves responsible for hypersensitivity in the gut and thus treat intestinal spasms [45]. Coumarin was present in all fractions apart from hexane with the highest yield found in ethyl acetate fraction (7.6±0.4 mg/g), followed by chloroform (5.2±0.3 mg/g), aqueous (3.8±0.2 mg/g) and crude extract (5.9±0.4 mg/g). Its identity was confirmed using a base ion at m/z 148 and fragments ions at m/z 105, 92 and 77 due to its lactone ring structure [46]. Coumarin is an inhibitor of L-type calcium channels that causes dose-dependent relaxation of the gastrointestinal smooth muscles similar to verapamil. Coumarin also inhibits pro-inflammatory cytokines (TNF-α, IL-6) through NF-κB and MAPK pathways [47]. It can therefore explain why both cinnamaldehyde and coumarin contribute significantly to the synergistic spasmolytic and antidiarrhoeal effects.

The synergistic combination of cinnamaldehyde and coumarin was additionally substantiated by FTIR spectroscopic results. The synergistic crude extract exhibited an intensely broad peak at 3270 cm^{-1} (O–H stretching of phenolic hydroxyl group). This peak is usually associated with polyphenol-rich plant extracts which provide better radical scavenging effect and mucosal membrane protective activity (Shakeel et al., 2025). The strong peak at 1702 cm^{-1} is an indication of C=O stretching of ketone, aldehyde (such as cinnamaldehyde), or carboxylic acid, compounds possessing antimicrobial and anti-inflammatory properties [48]. The sharp peak at 1608 and 1512 cm^{-1} shows aromatic C=C ring stretching, a common feature of conjugated molecules like curcumin and berberis. The chloroform extract was characterized by moderate O-H peak at 3380 cm^{-1}, intense C=O (1698 cm^{-1}), C=C aromatic peaks at 1600 and 1505 cm^{-1}, and C-H bending peak at 1458 cm^{-1}, indicating alkaloid (berberine) and terpenoid content. In the ethyl acetate extract, the strongest peaks were those corresponding to functional groups, including the O-H band (3300–3400 cm^{-1}), C=O peak (1705 cm^{-1}) typical for tannins, aromatic C=C peaks at 1605 and 1510 cm^{-1} for flavonoids, C-O stretching peak (1270 cm^{-1}) for phenolic esters, and the peak at 1040 cm^{-1}, confirming glycosidic bonds. These data confirm the ethyl acetate fraction's high biological activity.

Analysis by high-resolution NMR spectroscopy (^{1}H-NMR, ^{13}C-NMR, COSY, and HSQC) of the chloroform and ethyl acetate extracts confirmed the structural composition of the compounds. The ^{1}H-NMR spectrum of the chloroform extract displayed aromatic peaks from 6.5 to 8.0 ppm, with sharp peaks at 7.60 (s, 1H), 7.50 (d, 1H), and 6.85 ppm (s, 1H). Singlets within the methoxy region (3.80 to 4.00 ppm; 6H) denote tetraoxygenated berberine-like alkaloids. The ethyl acetate extract revealed a highly complicated aromatic spectral region (6.0 to 8.0 ppm), which included a singlet at 7.70 ppm and doublets at 7.20 ppm and 6.90 ppm, suggesting a characteristic configuration of olefinic protons of curcuminoids in the trans position. The sharp singlet signal at 9.65 ppm denoted the aldehyde proton of cinnamaldehyde. In the case of the ^{13}C-NMR spectrum, one particular resonance was noted at 182.5 ppm. It is associated with the enolic carbonyl group of curcumin, which is necessary for its metal-chelating and antioxidant properties [49]. Cross-peaks at 7.70 and 6.90 ppm, thus proving that the cinnamaldehyde side chain had a trans structure. HSQC correlated protons' frequencies with their respective directly bonded carbon atoms, thus confirming CH, CH_2, and CH_3 environments [50].

On the basis of the research, a recommendation is made that there is need to isolate and characterize the most active compounds especially in ethyl acetate and chloroform fractions with a view of developing them into standardized herbal formulations. Future studies should entail further toxicity profiling (acute and chronic), pharmacokinetics, and clinical trials to determine toxicity and efficacy in humans. Further, an isobolographic analysis of the precise

interaction of the Curcuma cassia and Berberis lyceum, either being additive, synergistic or potentiating could also offer greater insight into the synergistic effect. Research into how they affect role gut microbiota and ion channel targets (e.g., CFTR, K ions channels) are also encouraged. Lastly active fractions should be standardized on marker compounds (e.g., berberine, curcumin) so that there is consistency batch-to-batch and a certainty of therapeutic efficacy.

9. CONCLUSION: This study demonstrates that HPLC, GC-MS, FTIR, and NMR analyses confirmed the presence of bioactive compounds including berberine, curcumin, cinnamaldehyde, flavonoids, and tannins in the extracts. Among the solvent fractions, the ethyl acetate and chloroform fractions exhibited the highest concentrations of these bioactive constituents. As long as the researcher is interested in a particular class of chemicals, the procedures outlined under each class of compounds can be used to other kinds of plants. For the qualitative evaluation of secondary metabolites in a mixture, LC-MS/MS is unique due to the broad polarity range of compounds it covers. Although derivatization is required for polar molecules to study them using GC-MS, GC-MS may offer a greater resolution. Future studies should entail further toxicity profiling (acute and chronic), pharmacokinetics, and clinical trials to determine toxicity and efficacy in humans.

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12. Conflict of interest disclosure: The author declares that this research has no conflict of interest.
13. Permission to reproduce material from other sources: No material was reproduced in this study from other sources

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