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Original Article

Simplified Column Fractionation with DPPH Assay Guidance: Isolated a Potent Hepatoprotective Fraction from *Cassia auriculata*

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ABSTRACT

Background: Hepatoprotection is a critical area for research due to limitations of synthetic drugs and their side effects in the treatment of liver diseases. Natural compounds, particularly those derived from herbs with hepatoprotective therapeutical potential are serving as a best alternative for the same indication. *Cassia auriculata*, used traditionally in Indian folk medicine, has having a promising pharmacological activities, including hepatoprotective effects. **Objectives:** Present study aimed to evaluate using antioxidant activity to find out hepatoprotective compounds present in *C. auriculata* leaf extract and its solvent fractions through *in vitro, in vivo* experiments and LCMS analysis. **Materials and Methods:** The DPPH assay was employed to assess *in vitro* antioxidant activity, while cytotoxicity and hepatoprotective effects against H_2O_2-induced hepatotoxicity in HepG2 cell lines were investigated. Furthermore, the hepatoprotective activity of the most active Column fraction was evaluated against CCl_4-induced hepatotoxicity in albino Wistar rats. LCMS analysis was utilized to identify bioactive compounds in the most active Column fraction and correlations between antioxidant activity and phenolic, tannin and flavonoid contents were also explored. **Results:** The findings of the present study suggest that most active Column fraction from *C. auriculata* possess significant antioxidant and hepatoprotective properties, making them promising candidates for further investigation as potential therapeutic agents for liver diseases. **Conclusion:** This study aims to bridge the gap in understanding the correlation between antioxidant and hepatoprotective potential of *C. auriculata* extract and its fractions through rigorous experimental investigations. Overall, the present study contributes to the growing body of evidence supporting the therapeutic potential of natural compounds in liver disease management, paving the way for further research and development in this field.

Keywords: *Cassia auriculata*, Hepatoprotective activity, LCMS, HepG2 cell lines, Column chromatography.

INTRODUCTION

In the field of research there is a growing interest in hepatoprotection due to potential of natural compounds in treating the liver disease, as evidenced by extensive research on the medicinal plant constituents and hepatoprotective properties.[1] To treat liver disease synthetic compounds are associated with various side effects, leading to shift towards exploring natural compounds as safer alternatives.[2] Hepatoprotective agents, from the natural source are rich in triterpenes, flavonoids and polyphenols, having a most powerful protective agents in experimental liver-injury models.[3] Additionally, medicinal plants and their secondary metabolites having anti-oxidative, antifibrotic, anti-inflammatory, antitoxin and anti-cancerous mechanisms.[4] Furthermore, hepatoprotective properties of natural compounds such as silymarin, phyllanthin, hypophyllanthin, etc. have been most studied, highlighting the growing interest in harnessing the therapeutic potential of natural products.[5]

Cassia auriculata, commonly called as "Tanner's cassia" has been traditionally used in Indian folk medicine for its anti-diabetic Property.[6] Research has shown that the extracts of leaves and roots of *C. auriculata* possess hepatoprotective effects, protecting against oxidative stress and hepatotoxicity induced by galactosamine, ethanol and antitubercular drugs.[7,8] These effects may be attributed to the bioactive components present in the plant, such as glycosides, phenolic and other phytochemicals. Additionally, this plant has extensively studied for its antimicrobial, anti-inflammatory, antioxidant and anti-ulcer activities and it

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highlights a potential therapeutic benefits.[9-11] Presence of rich amount of bioactive compounds and diverse pharmacological activities make it a promising source for further exploration in the development of hepatoprotective agents.

DPPH assay is a commonly used method to evaluate antioxidant activity of the natural compounds.[12] This assay evaluates the ability of compounds to scavenge DPPH radicals, reflecting their free radical scavenging activity. Many researchers found correlation between the total phenolic content, total flavonoid content and the DPPH assay, which means that phenolic and flavonoid compounds are responsible for the antioxidant activity.[13] Additionally, the DPPH assay has been utilized to evaluate the antioxidant activity of various natural compounds, including flavonoids, xanthones, phenolic compound and other phytochemicals, demonstrating their potential as free radicascavengers.[14-16] Moreover, the DPPH assay has been employed to evaluate the antioxidant and hepatoprotective activities of compounds, underscoring its relevance in evaluating the potential hepatoprotective effects of natural bioactive compounds.[17,18] Therefore, the DPPH assay serves as a valuable tool for correlating the antioxidant activity of natural compounds with their potential hepatoprotective effects.

Column fractionation in natural medicine involves the separation and isolation of bioactive compounds from complex mixtures obtained from natural sources, such as medicinal plants, using column chromatography techniques.[19] This process is crucial for identifying and characterising the chemical entities responsible for the therapeutic properties of traditional medicines and natural products. The shortage of natural medicinal plants has led to the need to discover new resources to replace them and protect endangered natural plant species, making column fractionation an essential tool in the search for novel bioactive compounds.[20] Bioactivity-guided fractionation is a way for researchers to find active compounds before they are separated. It helps them get around the problems that come up when they try to separate bioactive compounds during column fractionation.[21] Utilising separation techniques based on molecular characteristics and interaction types, such as ion exchange, surface adsorption, partition and size exclusion, can aid in achieving effective compound separation.[22] Additionally, researchers can consider the influence of alternative liquid chromatography techniques on the chemical complexity and bioactivity of isolated mixtures, as well as the use of vacuum and open column strategies for separation, which can yield distinctive shifts in fraction profiles and biological activity.[23] Furthermore, the stepwise elution of compounds from one column to another can aid in achieving effective separation without further compound degradation.[24] Combining the benefits of online and offline screening methodologies, such as 1536 well micro-fractionation, can allow for fast fraction collection while maintaining high resolution achieved by the liquid chromatography separation.[25,26] Finally, bioactivity-directed fractionation and the testing of all column fractions for specific bioactivity can aid in the isolation of active compounds.[27,28]

The present study aims to employ simplified column fractionation guided by DPPH assay to isolate a potent hepatoprotective fraction from *Cassia auriculata* extract. The objective of the study is to evaluate the antioxidant and hepatoprotective properties of *Cassia auriculata* extract and its solvent fractions through *in vitro* and *in vivo* experiments. It aims to assess the scavenging activity of the extract and fractions using the DPPH assay and investigate their cytotoxicity and hepatoprotective effects against H_2O_2-induced hepatotoxicity in HepG2 cell lines. Additionally, the study intends to evaluate the hepatoprotective activity of the most active column fraction against CCl_4-induced hepatotoxicity in albino Wistar rats. An effort is also made to identify bioactive compounds in the *C. auriculata* fraction using LCMS analysis and explore the correlation between the found antioxidant activity and phenolic, tannin and flavonoid contents.

MATERIALS AND METHODS

Materials

Dr. Ganesh Babu, Assistant Professor, TDU (The University of Trans-Disciplinary Health Sciences and Technology), FRLHT, Bangalore, authenticated fresh aerial parts of *Cassia auriculata* (L.) collected from Nagamangala Taluk (Kanghatta), Mandya district, Karnataka, India.

A 20:80 (Water:ethanol) hydroalcoholic solution was used for extraction in the study. The DPPH assay was used to assess antioxidant activity, using quercetin and ascorbic acid as standards. Bioactive substances were estimated using Folin - Ciocalteu reagent (Sigma-Aldrich) for phenolics, aluminum chloride (Merck) for flavonoids and vanillin sulphuric acid (Fisher Scientific) for tannins. Using MTT (Sigma-Aldrich), HepG2 cell lines were cytotoxically tested *in vitro*. Petroleum ether (Sigma-Aldrich), ethyl acetate (Fisher Scientific), n-butanol (Thermo Fisher Scientific) and water solvents were used for liquid-liquid fractionation. For ethyl acetate, silica gel column chromatography was used. Thin Layer Chromatography (TLC) with toluene, acetone and formic acid mobile phases and UV-visible spectroscopy identified compounds. CCl_4 for inducing hepatotoxicity in, Wistar albino male rats and a hematoxylin and eosin staining kit (Abcam) were used in the *in vivo* hepatoprotective investigation.

Preparation of an aqueous extract

After the authentication of *Cassia auriculata* leaves, the aerial portion was removed and cleaned under running tap water to remove dust, dirt and other contaminants. The leaves dried in the shade for 25 days. The leaves were then processed into a fine powder in a kitchen grinder. Ultrasound-assisted leaf sample extraction involves mixing 500 g of powdered sample with

20:80 (water:ethanol) in a 1:10 drug-to-solvent ratio. Four times sonication conducted for a period of 15 min at room temperature (37.2°C). This temperature was maintained throughout the sonication method to minimize overheating and keep the solution around room temperature. Sonicated extracts were filtered through Whatman filter paper to remove solid debris. Rota vapour was employed to remove solvent from the extracts and to obtain concentrated extracts.[29,30]

Liquid-Liquid Fractionation of the Hydroalcoholic Extract of *Cassia auriculata* Leaves

To separate substances with different chemical properties by liquid-liquid fractionation, petroleum ether, ethyl acetate, n-butanol and water were used in ascending order of polarity.[31] In order to prepare the fraction, 50 g of hydroalcoholic extract was dissolved in 300 mL of water started solvent-solvent fractionation. To extract all constituents, similar fractions were pooled and each solvent was treated twice. After filtering and heating in a 50°C water bath, fraction residues were weighed and yields estimated. This methodical methodology separates phytoconstituents of similar properties and identifies an enriched fraction with the highest antioxidant activity, allowing column separation and comprehensive analysis of its physiologically active components.

DPPH Assay of *Cassia auriculata* Leaf Extract and Its Fractions

In accordance with Banskota AH *et al.* (2000),[32] 80 µL of DPPH solution, various concentrations of the test solution (25, 50, 100, 200, 400 and 800 µg/mL) and 240 µL of HPLC-grade methanol were combined. The reference standard concentrations were 0.3125, 0.625, 1.25, 2.5, 5 and 10 µg/mL. The reaction mixture was incubated at 25°C for 15 min and measured at 510 nm by a semi-autoanalyzer. Using nonlinear regression analysis in Graph Prism 5.0, half maximum Inhibitory Concentration (IC_{50}) was calculated.

The sample's relative activity was estimated by comparing the IC_{50} value to the standard and calculating the percentage inhibition. Lower activity is indicated by higher IC_{50} values.

Estimation of Bioactive Compounds in Fractions

The methodology for estimating bioactive compounds in fractions involves three assays: Total Phenolic Content, Total Flavonoid Content and Total Tannin Content. For Total Phenolic Content, the Folin-Ciocalteau reagent is employed, with absorbance measured at 765 nm and results expressed as mg GAE/g dry extract.[33] Total Flavonoid Content is determined using the aluminum chloride reagent method, with absorbance recorded at 510 nm and results expressed as mg CAE/g dry extract.[34] Total Tannin Content is assessed via the Vanillin-HCl method, with absorbance recorded at 500 nm and results expressed as mg CAE/g dry extract.[35] All measurements are conducted in triplicate and standard solutions are utilized to construct calibration curves for accurate quantification.

Purification of Ethyl Acetate Fraction from Hydroalcoholic Extract of *C. auriculata* Leaves via Column Chromatography

The ethyl acetate fraction from the hydroalcoholic extract of *C. auriculata* leaves was purified via column chromatography, owing to its exceptional antioxidant capacity and minimal cytotoxicity against HepG2 cell lines as demonstrated by the MTT assay. A Borosil glass column, specifically designed with dimensions of 62 cm x 4.5 cm x 4.5 cm, was packed with 500 g of silica gel (mesh size: 200-400). The column was wet-packed with activated silica gel slurry in petroleum ether solvent, ensuring a uniform packing and minimal voids.[36] After an initial cotton wool coating at the bottom of the column, the silica gel slurry was poured slowly and continuously while tapping the column to prevent air pockets. The column was then conditioned with petroleum ether solvent for 2 hr prior to sample loading. The sample was mixed in a 1:1 ratio with activated silica gel to form a sample bed atop the stationary phase and fractionation was carried out using gradient elution. Petroleum ether was initially used as the solvent at a flow rate of 15 drops per minute, followed by elution with chloroform, chloroform-ethyl acetate mixtures and ethyl acetate-methanol mixtures in various ratios. Table 1 details the volumes of each mobile phase used for fraction collection, encompassing a comprehensive overview of the elution process and facilitating chemical extraction from the ethyl acetate fraction. In total, 39.05 L of fractions were collected across 17 different fractions, each containing 50 mL.

Thin Layer Chromatography (TLC) Analysis of Ethyl Acetate Fraction and Column Fractions from *Cassia auriculata*

The TLC analysis of the ethyl acetate fraction and column fractions from *Cassia auriculata* involved specific procedures tailored for each. Initially, TLC was conducted on the ethyl acetate fraction using Toluene: Acetone: Formic acid (6:6:1) as the mobile phase, selected for its optimal separation, development duration and band count. TLC plates were meticulously prepared and observed under visible, short and long-wavelength UV light to visualize the bands, with each band's Retardation factor (R_f) calculated. Subsequently, the TLC plate was stained with DPPH reagent (3 mg DPPH in 100 mL methanol) to detect antioxidants and new R_f values were determined post-air drying.[37]

For the pooling of collected fractions, Thin Layer Chromatography was performed on pre-coated Silica Gel GF254 TLC plates using Acetone: Toluene: Formic acid (6:6:1) as the mobile phase, with DPPH utilized as the visualization reagent. This facilitated the identification of antioxidants. Following DPPH staining, TLC fingerprints were used to pool fractions, which were then dried and stored in sealed containers for further analysis. This integrated

technique offers a comprehensive examination of the ethyl acetate fraction and aids in the identification of antioxidant compounds present within the fractions, enhancing the understanding of the sample's chemical composition and potential bioactivity.

In vitro Cytotoxicity Assay (MTT) of Fractions of Hydroalcoholic Extracts of *Cassia auriculata* on HepG2 Cell Lines

Hydroalcoholic extract was fractionated using different-polarity of solvents. DPPH and MTT assays on HepG2 cell lines assessed the fractions' antioxidant capacity. Drug toxicity was assessed by evaluating cell proliferation and survival. A 96-well plate was used to seed HepG2 cells, which were treated with varied doses of chemicals for 48 hr for the MTT experiment. After 4 hr, Thiazolyl Blue Tetrazolium Bromide dissolved formazan crystals for 570 nm spectrophotometric examination. For dose-dependent responses, Megalan and GraphPad Prism 8.2 computed IC_{50} values from absorbance data. The histogram data, based on at least three tests, revealed *Cassia auriculata* hydroalcoholic fractions' cytotoxicity on HepG2 cell lines.[38,39]

In vivo Hepatoprotective Activity of C2 Column Fraction by CCl_4 Animal Model

Healthy Wistar albino male rats (200-250 g) underwent a week of acclimatization to laboratory conditions. Housed in clean cages with a standard diet and water ad libitum, under a 12/12 hr dark/light cycle at Sri Adichunchanagiri College of Pharmacy, B.G. Nagara, the experiments followed CPCSEA guidelines.

Animals were randomly divided into six groups (n=6), the treatment protocol is detailed in Table 2. The normal control received oral saline, disease control was treated with CCl_4 on the 7th day and the standard group received oral Silymarin. Test doses (50, 100, 150 mg/kg) of C2 Column Fraction were administered orally for 7 days, followed by CCl_4 induction on the 7th day. On the 8th day, animals were sacrificed and liver sections were stored in formalin. Blood samples were analyzed for liver function tests, including SGPT, SGOT, ALP, Serum bilirubin and Total proteins.

For histological analysis, liver sections were processed, embedded in paraffin, cut into 5 µm thick sections and stained with Haematoxylin and Eosin (HE). This comprehensive approach assessed the hepatoprotective activity of the C2 Column Fraction in the CCl_4-induced hepatotoxicity model.[40-42]

LCMS Analysis

The LCMS analysis was conducted using a Waters Acquity UPLC system coupled to a Waters Xevo G2-XS QT of mass spectrometer. A column of Accucore C18 (50 x 4.6, 2.6 µ) was employed for chromatographic separation. Prior to analysis, the mobile phase was prepared by adding 0.1% formic acid in water as Mobile Phase-A, while Mobile Phase-B consisted of acetonitrile. Samples, including the C2 fraction, were injected at a volume

Table 1: Fraction collection data.

Sl. No.	Name of the fractions	Number of fractions collected (50 mL each)	Total volume of fraction collected in L
F1	Petroleum ether: Chloroform (100:0).	20	1
F2	Petroleum ether: Chloroform (80:20).	20	1
F3	Petroleum ether: Chloroform (60:40).	38	1.9
F4	Petroleum ether: Chloroform (40:60).	30	1.5
F5	Petroleum ether: Chloroform (20:80).	15	0.75
F6	Petroleum ether: Chloroform (0:100).	12	0.6
F7	Chloroform: Ethyl acetate (95:5).	24	1.2
F8	Chloroform: Ethyl acetate (90:10).	50	2.5
F9	Chloroform: Ethyl acetate (75:25).	31	1.55
F10	Chloroform: Ethyl acetate (50:50).	66	3.3
F11	Chloroform: Ethyl acetate (25:75).	50	2.5
F12	Chloroform: Ethyl acetate (20:80).	43	2.15
F13	Chloroform: Ethyl acetate (10:90).	95	4.75
F14	Chloroform: Ethyl acetate (0:100).	84	4.2
F15	Ethyl acetate: Methanol (90:10).	67	3.35
F16	Ethyl acetate: Methanol (80:20).	98	4.9
F17	Ethyl acetate: Methanol (70:30).	38	1.9
Total volume of fraction collected= 39.05 L			

of 5 µL. The chromatographic separation was achieved using a gradient program. The gradient elution profile was represented in Table 3.

During analysis, the capillary voltage was set to 3.0 KV, collision energy to 20 V (with a ramp from 30-90V), source temperature at 150ºC, desolvation temperature at 450ºC and cone gas flow at 50 L/Hr.

The LCMS analysis provided comprehensive results, facilitating the identification of compounds of interest, including those present in the C2 fraction, in the samples.

Statistical Analysis

The experimental results' reliability and validity depended on this study's statistical analysis. The antioxidant potential of *Cassia auriculata* extract and its solvent fractions was assessed using One-way Analysis of Variance (ANOVA) to investigate DPPH radical inhibition. Statistical significance of observed differences in DPPH inhibition among fractions was determined using post-hoc Dunnett's comparison tests, with a significance threshold of $p<0.05$. In the *in vitro* cytotoxicity assay, one-way ANOVA and Student's t-test were used to determine the significance of the ethyl acetate fraction's protective effects against H_2O_2-induced hepatotoxicity in HepG2 cell lines. For the *in vivo* assessment of CCl_4-induced hepatotoxicity, biomarker changes were analyzed using one-way ANOVA and Dunnett's comparison tests. A significance level of $p<0.05$ was used to compare treatment groups and the disease control group. To assess the significance of observed improvements in liver architecture and attenuation of CCl_4-induced hepatic lesions, liver tissues were analyzed using statistical methods like chi-square tests or Mann-Whitney U tests, with a significance level of $p<0.05$. By systematically employing statistical analysis throughout the investigation, experimental results and conclusions were reliable and legitimate.

Table 2: Grouping of Animals.

Groups	Treatment Protocol
Group-I	Normal control: (Saline 1 mL/Kg body weight p.o for 7 days).
Group-II	Disease Control: Saline 1 mL/Kg body weight p.o for 6 days+(CCl_4: olive oil [1:1] 1 mL/animal intraperitonially on 7th day).
Group-III	Standard (Silymarin 100 mg/Kg body weight p.o for 7 days)+CCl_4: olive oil [1:1] 1 mL/animal intraperitonially on 7th day post treatment.
Group-IV	Test dose-1: Column fraction 50 mg/Kg., *p.o* for 7 days)+CCl_4: olive oil [1:1] 1 mL/animal intraperitonially on 7th day post treatment.
Group-V	Test dose-2: Column fraction 100 mg/Kg., *p.o* for 7 days)+CCl_4: olive oil [1:1] 1mL/animal intraperitonially on 7th day post treatment.
Group-VI	Test dose-3: Column fraction 150 mg/Kg., *p.o* for 7 days)+CCl_4: olive oil [1:1] 1 mL/animal intraperitonially on 7th day post treatment.

RESULTS

Preparation of Aqueous Extract and Liquid-Liquid Fractionation

The aqueous Extract of *Cassia auriculata* (CAE) was meticulously prepared following a systematic methodology, resulting in a reddish-brown colored extract with a percentage yield of approximately 35.78%.

Furthermore, the liquid-liquid fractionation of the hydroalcoholic extract yielded four distinct fractions, each with unique characteristics as outlined in Table 4. The fractions included the pet. ether fraction, ethyl acetate fraction, n-butanol fraction and aqueous fraction, with yields of 1.5%, 37.7%, 10.38% and 30.84% respectively. Each fraction exhibited different colors and textures, ranging from green semi-solid to dark brown solid, greenish-brown powder and brown powder. This dual approach of aqueous extract preparation and liquid-liquid fractionation not only successfully obtained the CAE but also provided distinct fractions for further investigation of their composition and potential biological activities.

DPPH Assay of *C. auriculata* Extract and its Solvent Fractions

The results of the DPPH radical scavenging assay for *Cassia auriculata* extract and its solvent fractions, along with the

Table 3: LCMS Analysis Gradient Elution Profile.

Time	Flow (mL)	%A	%B
Initial	0.500	90.0	10.0
1.00	0.500	90.0	10.0
6.00	0.500	50.0	50.0
12.00	0.500	5.0	95.0
16.00	0.500	5.0	95.0
17.00	0.500	90.0	10.0
20.00	0.500	90.0	10.0

standard Ascorbic acid, are visually depicted in Figure 1. Ascorbic acid served as the positive control, exhibiting a % inhibition of 47.44±1.09. Remarkably, the *C. auriculata* extract demonstrated significant antioxidant potential with a % inhibition of 81.83±1.41, surpassing the standard. Among the solvent fractions, the ethyl acetate fraction stood out with the highest % inhibition at 95.25±0.47, indicating a robust antioxidant activity. In contrast, the pet. Ether and aqueous fractions displayed lower DPPH scavenging activities at 16.79±0.35 and 14.95±0.28, respectively. The n-butanol fraction also demonstrated substantial antioxidant activity, registering a % inhibition of 90.637±0.495.

Comprehensive Evaluation of Ethyl acetate fraction Using DPPH Bioautography and TLC Method with Varied Mobile Phases

The incorporation of both DPPH assay and TLC method with seven different mobile phase systems in the separation process enhances the comprehensive understanding of *Cassia auriculata* extract. Notably, Toluene: Acetone: Formic acid (6:6:1) as effective mobile phase systems for their ability to provide good resolution and distinguish multiple chemical entities. This selection is crucial for guiding the subsequent column separation process. The assessment under UV 366 nm and 254 nm, along with the application of DPPH reagent and ferric chloride reagent, facilitates a multi-dimensional analysis of the compounds. The observed separation and R_f values mentioned in the Table 5 and Figure 2, contribute valuable insights, paving the way for targeted isolation and further exploration of the bioactive constituents present in *Cassia auriculata*.

Total Phenolic Content (TPC), Total Tannin Content (TTC), Total Flavonoid Content (TFC) and DPPH Assay Correlation Analysis

The Table 6 presents the results of various content analyses for different sample fractions. Total Phenolic Content (TPC) ranges from 156.8±0.38 mg GAE/g in the aqueous fraction to 952.31±1.21 mg GAE/g in the ethyl acetate fraction, indicating significant variations in phenolic compounds across the fractions. Total Tannin Content (TTC) also varies notably, with the highest value of 556.02±0.28 in the ethyl acetate fraction and the lowest value of 15.89 in the aqueous fraction. Similarly, Total Flavonoid Content (TFC) demonstrates considerable diversity, with the highest value of 217.07±0.97 CAE/g equivalent in the ethyl acetate fraction and the lowest value of 100.73±0.72 CAE/g equivalent in

Table 4: Physical charectristics of the fractions.

Fraction characteristic's	Pet. ether fraction	Ethyl acetate fraction	n-butanol fraction	Aqueous fraction
Fraction Yield	1.5%	37.7%	10.38%	30.84%
Colour and texture of the fraction	Green semi solid.	Dark brown solid.	Greenish brown powder.	Brown powder.

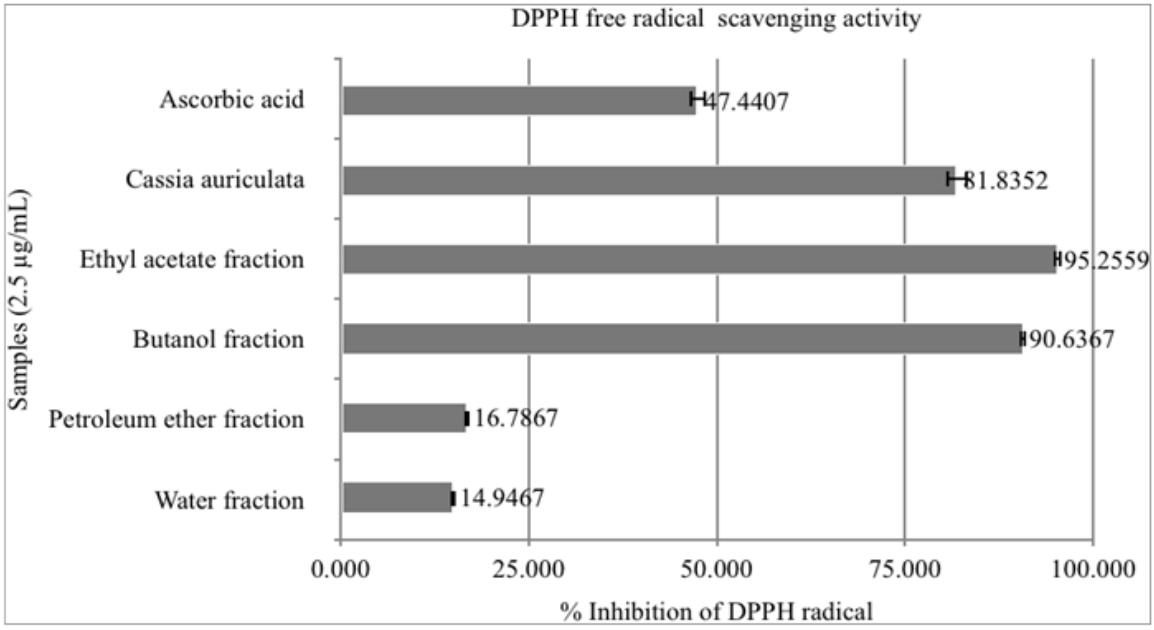

Figure 1: DPPH free radical activity of Ascorbic acid, *Cassia auriculata* and its fractions.

the n-butanol fraction. These findings suggest distinct chemical compositions and antioxidant capacities among the analyzed fractions, highlighting the importance of fractionation in studying bioactive compounds in natural products.

DPPH free radicals scavenging activity

The DPPH free radical scavenging activity of all the 16 pooled fractions and the Ethyl acetate fraction was performed and Inhibitory Concentration-50 (IC_{50}) values were assessed. As seen in the below Table 7 all the collected fractions exhibited concentration dependent percentage inhibition of DPPH free radicals, the same is depicted in the graphs in the Table 7.

Column Fractions Pooling and DPPH Free Radicals Scavenging Activity

In the process of column elution, fractions were systematically collected and assessed for antioxidant compounds, leading to the creation of 16 distinct pooled fractions (Fraction A to Fraction O) identified through TLC pattern similarities. The pooling involved

Table 5: Development of TLC method for different fraction.

Sl. No.	Sample	R_f Value			
		UV 366	DPPH	UV 254	$FeCl_3$
1	CAE	0.11 0.2 0.43 0.55 0.7 0.95	0.05 0.16 0.36 0.45 0.55 0.61	0.11 0.43 0.55 0.7 0.95	0.11 0.43 0.55 0.7 0.95
2	Pet. Ether fraction	0.7 0.86 0.95	-	0.95	-
3	Ethyl acetate fraction	0.55 0.7 0.95	0.38 0.45 0.55	0.55 0.7 0.95	0.46 0.65
4	n-Butanol fraction	0.11 0.43 0.55 0.7 0.95	0.11 0.4 0.48 0.53 0.6	0.2 0.61	0.2 0.61
5	Aqueous fraction	0.43 0.55 0.62 0.65 0.7	0.05	-	0.03

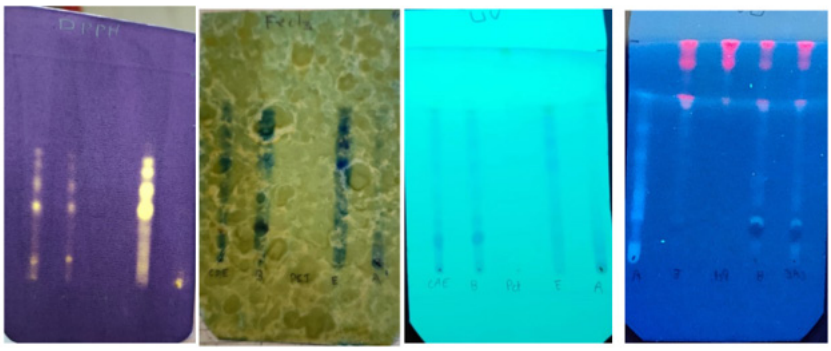

Figure 2: Development of TLC method for each method.

Table 6: Comparison of Total Phenolic Content (TPC), Total Tannin Content (TTC), Total Flavonoid Content (TFC), and DPPH Assay Correlation Analysis across Different Sample Fractions.

Samples	mg VCEAC/g dry extract	TPC (mg GAE/g dry extract)	TTC (mg CAE/g dry extract)	TFC (mg CAE/g dry extract)
CAE	224.25 ± 0.32^{c}	785.81 ± 0.83^{b}	63.18 ± 1.01^{b}	63.18 ± 1.01^{b}
Aqueous fraction	138.20 ± 0.54^{a}	156.8 ± 0.38^{a}	15.89 ± 0.48^{a}	15.89 ± 0.48^{a}
Petroleum ether fraction	168.81 ± 0.93^{b}	148.2 ± 1.04^{a}	114.98 ± 0.71^{c}	114.98 ± 0.71^{c}
Ethyl acetate fraction	285.46 ± 0.51^{e}	952.31 ± 1.21^{d}	556.02 ± 0.28^{e}	556.02 ± 0.28^{e}
n- Butanol fraction	266.27 ± 1.01^{d}	827.5 ± 0.89^{c}	265.3 ± 0.63^{d}	265.3 ± 0.63^{d}

VCEAC: vitamin C equivalent antioxidant capacity; **GAE:** Gallic acid equivalent; **CAE:** Catechin equivalent. Values in the same column with different alphabets indicates the presence of significant differences.

Table 7: Weight, DPPH radical scavenging values and Cytotoxicity Assessment of Ethyl Acetate Fractions.

Name of pooled fraction	Wt. of fractions in grams	DPPH radical scavenging values (IC_{50})	Percentage of cells viable (Control: 100, H_2O_2)
Ethyl acetate fraction		30.20 µg^{f}	73.89 ± 0.12^{f}
Fraction-A	0.36	113.04 µg^{k}	62.94±2.08^{d}
Fraction-B	2.67	25.20 µg^{c}	72.35±0.67^{f}
Fraction-C1	1.46	20.90 µg^{b}	84.12±1.16^{i}
Fraction-C2	12.76	**14.94 µg^{a}**	95.29±1.00^{l}
Fraction-D	1.58	25.25 µg^{c}	91.76±1.66^{k}
Fraction-E	2.03	27.34 µg^{e}	84.71±0.83^{j}
Fraction-F	0.61	27.33 µg^{e}	80.00±1.41^{g}
Fraction-G	1.66	26.59 µg^{d}	84.71±0.83^{i}
Fraction-H	0.76	32.10 µg^{g}	89.41±0.83^{j}
Fraction-I	0.81	67.03 µg^{h}	81.18±0.33^{h}
Fraction-J	0.42	98.02 µg^{j}	60.00±0.83^{d}
Fraction-K	0.29	84.37 µg^{i}	63.53±0.42^{d}
Fraction-L	0.37	127.21 µg^{l}	49.41±0.58^{c}
Fraction-M	0.15	**247.08 µg^{n}**	44.71±0.58^{b}
Fraction-N	0.81	197.67 µg^{m}	43.53±0.50^{b}
Fraction-O	2.02	158.49 µg^{k}	49.41±0.33^{c}
H_2O_2			37.65 ± 0.25^{a}

Values are represented as mean±standard deviation. Values in the same column with different alphabets indicates the presence of significant differences.

varying numbers of fractions, each contributing specific weights, as outlined in Table 7.

Subsequently, the DPPH free radical scavenging activity was scrutinized for both the Ethyl Acetate fraction and the 16 pooled fractions.

Concentration-dependent inhibitory responses were observed, with the Ethyl Acetate fraction displaying a notable percentage inhibition of 79.18% at 50 µg/mL. The calculation of IC_{50} values, reflecting the concentration required for 50% scavenging of DPPH free radicals, highlighted the distinct antioxidant capabilities of each fraction. Notably, Fraction-C2 emerged as the most potent, with an IC_{50} value of 14.94 µg, while Fraction-M demonstrated the least activity with an IC_{50} of 247.08 µg. These findings underscore the diverse antioxidant potential within the collected fractions, providing valuable insights for further exploration of bioactive compounds.

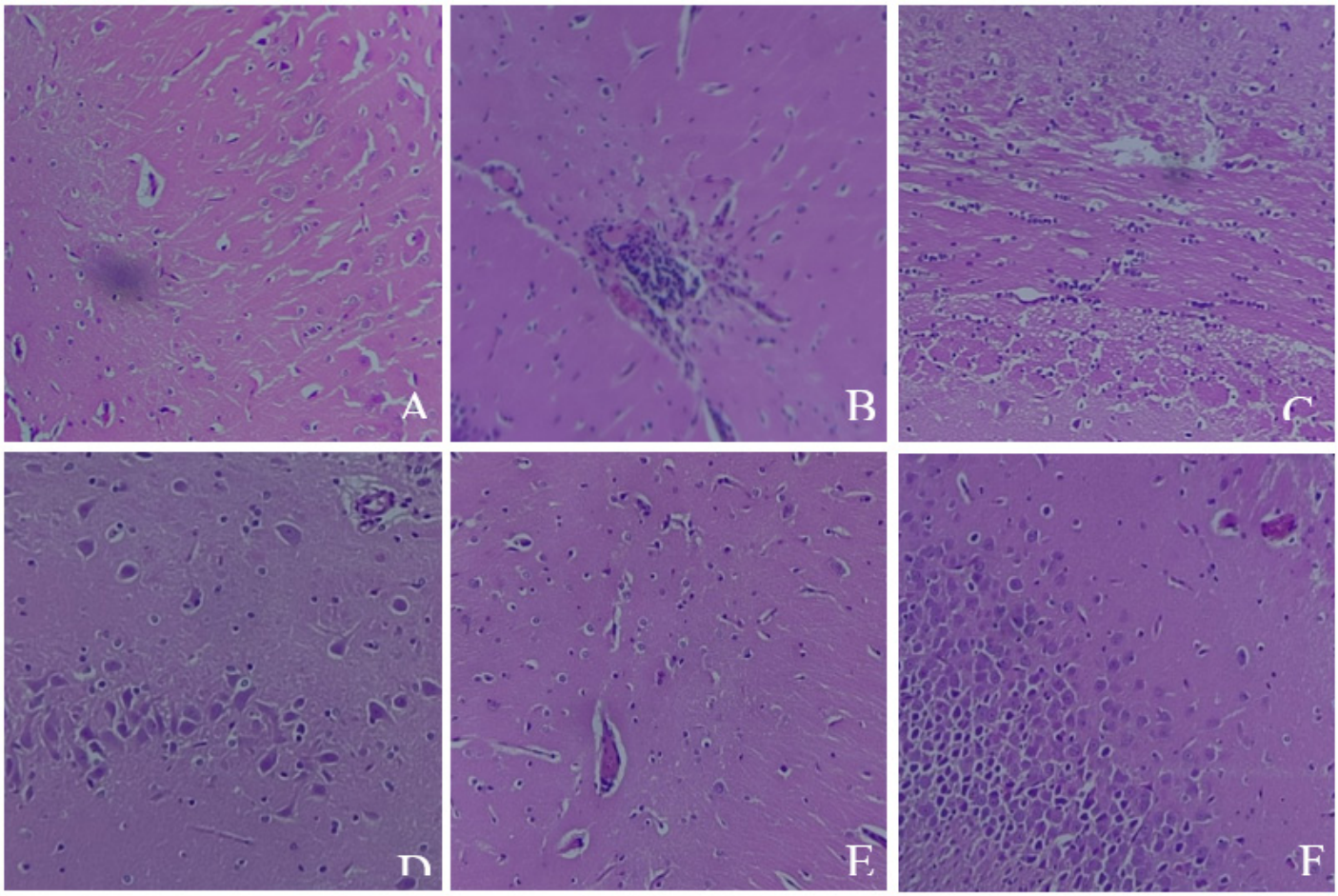

Figure 3: Representative sections A, B and C are sections of the liver of Normal control, Disease control and Silymarin treatment group stained with hemotoxin and eosin, sections D, E and F represent the liver sections of Animals treated with Column fractions C-2 at different concentrations.

Table 8: Effect of Silymarin and test doses.

Groups	SGOT in U/L	SGPT in U/L	ALP in U/L	Total proteins in mg/dL
Normal	38.3±1.4*	32.81±1.56*	264.4±1.98*	139.45±4.04*
Disease control	116.33±2.91*	67.48±2.71*	763.5±.341*	37.55±3.91*
Standard	40.41±1.36***	37.28±1.41***	481.4±3.94***	77.24±2.84***
Fraction 50 mg	90.33±0.95**	64.26±2.18**	644.25±3.67**	87.54±2.89***
Fraction 100 mg	77.3±1.16**	50.2±1.12**	537.55±2.42**	102.39±3.33**
Fraction 150 mg	59.16±2.14***	46.16±2.18**	417.21±2.21**	115.96±3.23**

Results are expressed as Mean±SEM of Six animals from individual groups, the results were analysed by One way ANOVA followed by Dunnett's comparision-tests. *represents significant at $p<0.05$, ** represents significant at $p<0.01$ and *** represents highly significant at $p<0.001$, all the values were compared with the disease control and were considered as statistically significant.

In vitro Cytotoxicity Assay (MTT) of Fractions of Hydroalcoholic Extracts of *Cassia auriculata* on HepG2 Cell Lines

The protective effect of different column fractions against H_2O_2 was determined using MTT assay and the results were expressed in terms of Percentage of cell viability as indicated in the Table 7. Exposure of Hydrogen peroxide to the HepG2 cells without the pretreatment of column fractions resulted in decrease in cell viability to 37.65±0.25%, the pretreatment of the cells with the Column fractions with 100 µg of the sample solution resulted in varied cell viability percentage with each column fractions, among the column fractions, column fractions C-2, D and H showed maximum protective effect against the damage caused by the H_2O_2, the treatment with Column fraction C2 showed maximum protective effect with 95.29±1.00% cell viability after treatment with hydrogen peroxide.

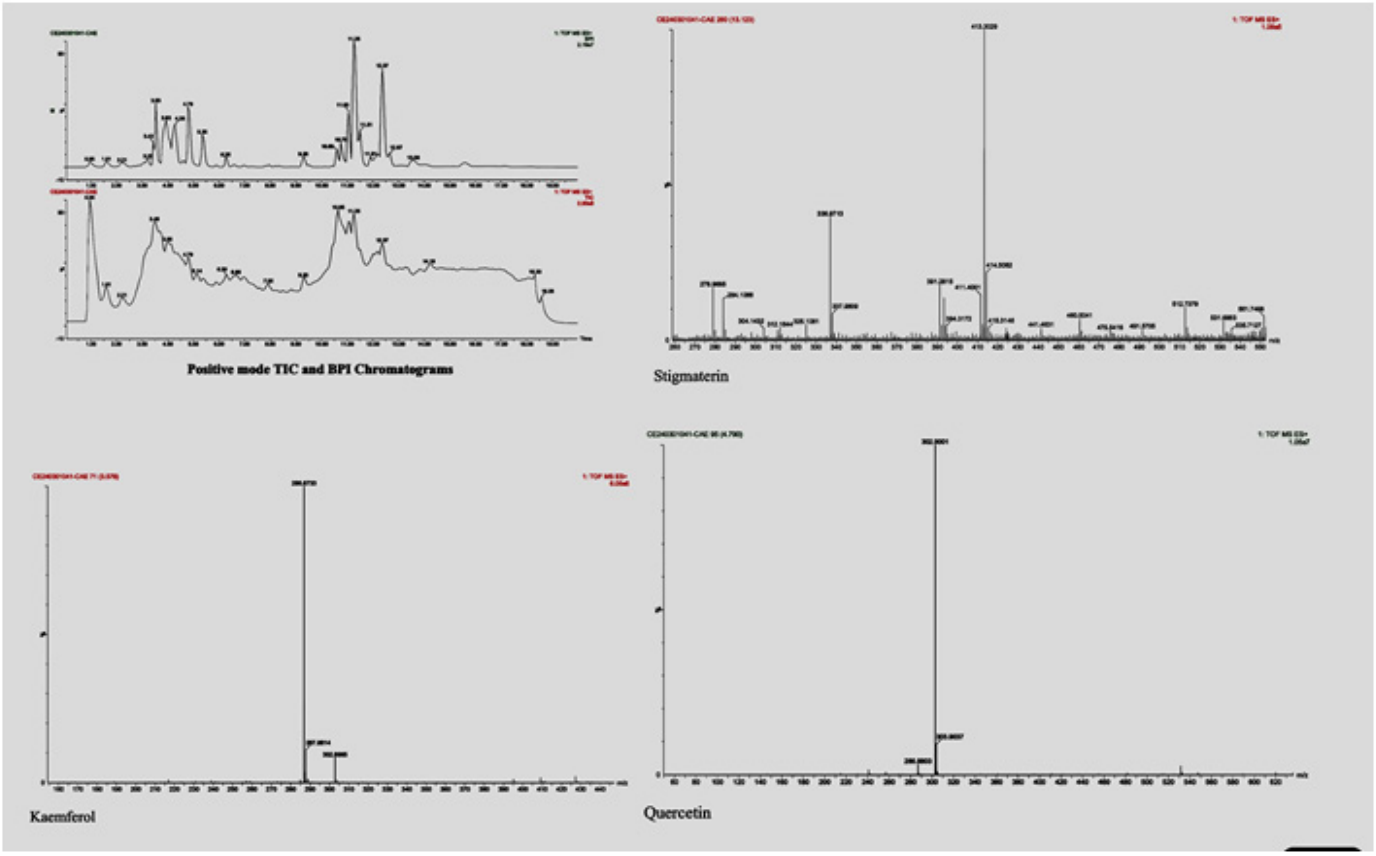

Figure 4: Positive mode TIC and BPI Chromatograms.

Table 9: Effect of C2 Column Fraction on bilirubin.

Groups	Total bilirubin	Direct bilirubin	Indirect bilirubin
Normal	1.39±0.22*	0.68±0.06*	0.7±0.18*
disease control	5.48±0.18**	2.97±0.15**	2.5±0.16**
standard	2.01±0.25***	0.99±0.12***	1.02±14***
fraction 50	5.15±0.16*	2.6±0.04*	2.54±0.16*
fraction 100	4.78±0.14*	2.36±0.04*	2.41 ±0.14**
fraction 150	3.5±0.17**	1.74±0.06*	1.75±0.12**

Results are expressed as Mean±SEM of Six animals from individual groups, the results were analysed by One way ANOVA followed by Dunnett's comparision-tests. *represents significant at $p<0.05$, ** represents significant at $p<0.01$ and *** represents highly significant at $p<0.001$, all the values were compared with the disease control and were considered as statistically significant.

In vivo hepatoprotective activity of C2 column fraction by CCl_4 animal model

CCl_4 Induced Hepatoprotective activity evaluation in Albino Wistar Rats

Following the results of the *in vitro* hepatoprotective activity evaluation of 16 column fractions obtained, Column fraction number C2 was selected for further *in vivo* hepatoprotective activity evaluation so as to extrapolate the extent of hepatoprotection conferred by the most active column fraction with that of actual hepatoprotection in animals, by using CCl_4 as hepatotoxin.

The Increased levels of Hepatic serum biomarkers such as SGOT, SGPT, ALP, Total Bilirubin, Direct and Indirect Bilirubin and decreased levels of Total proteins in CCl_4 treated animals in comparison to the animals in the Normal groups indicated the

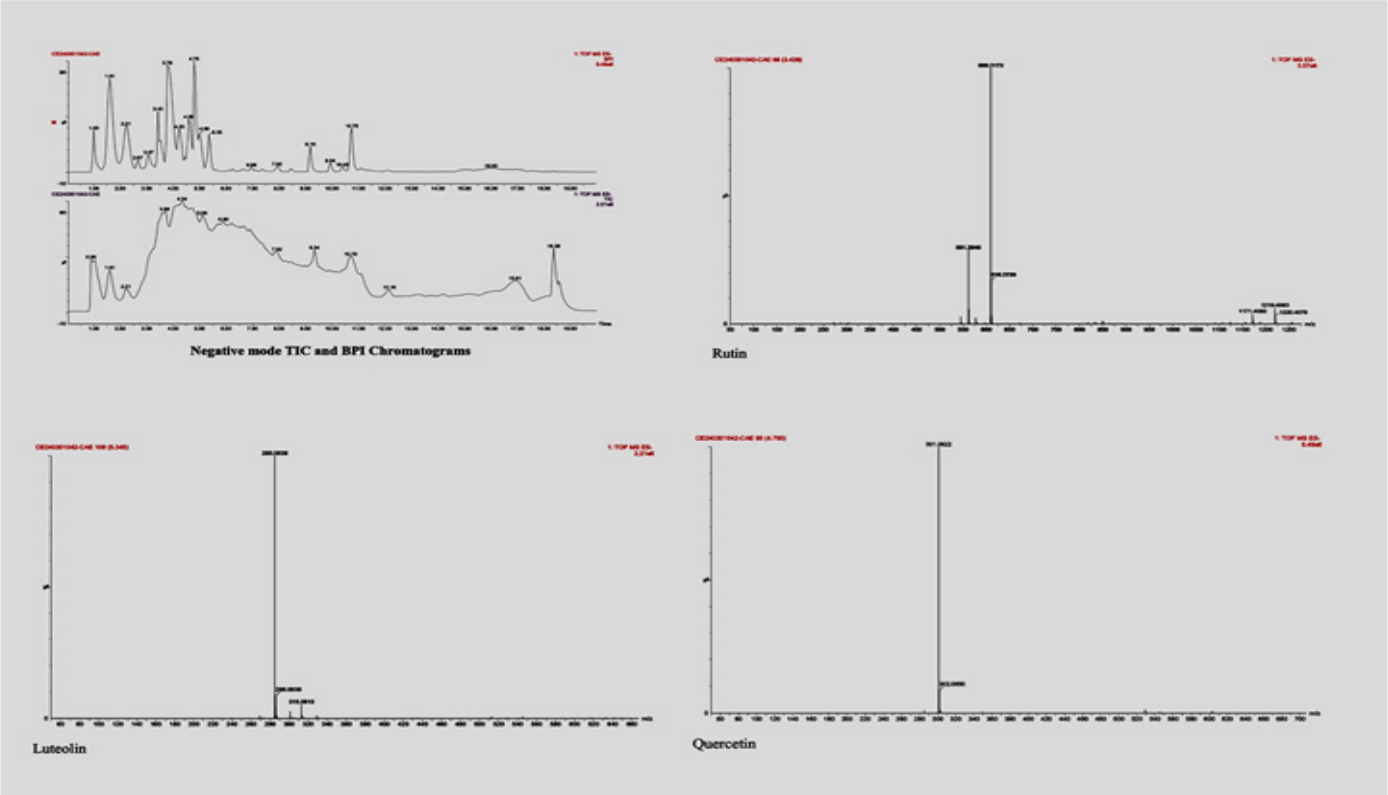

Figure 5: Chromatogram showing peak corresponding to stigmaterin.

successful induction of hepatic damage in the Experimental animals. Pre-treatment of animals with Silymarin had a significant effect on increased levels of SGOT, SGPT, ALP, Total Bilirubin, Direct and Indirect Bilirubin and Decreased levels of Total proteins and treatment with column fractions showed significant effects on the levels of serum bio markers with increase in the treatment doses of 50,100 and 150 mg/Kg body weight.

Effect of C2 Column Fraction on SGOT levels in CCl_4 induced Hepatotoxicity

Induction of hepatic damage with CCl_4 altered the levels of serum bio markers in the animals, the results in Table 8 indicate the levels of SGOT of the experimental animals, levels of SGOT in the Disease Control group is drastically increased compared to the normal levels of SGOT in the Normal group. Pre-treatment with Silymarin prevented the elevation of the SGOT levels remarkably. Pre-treatment of the fractions also tend to have a control over the increasing levels of SGOT in a dose dependent manner, Pre-treatment with 150 mg/Kg body weight had significant effect on the elevated levels and have comparable activity to that of Silymarin standard.

Effect of C2 Column Fraction on SGPT levels in CCl_4 induced Hepato-toxicity

CCl_4 Altered the levels of serum bio markers in the animals, the results in Table 8 represent the average levels of SGPT in animals of each group is radically elevated in comparison to the normal levels of SGPT in the Normal group. Pre-treatment of Silymarin barred the elevation of the SGPT levels. Pre-treatment of the Column fractions also showed control over the increasing levels of SGPT in a dose dependent manner.

Effect of C2 Column Fraction on ALP levels in CCl_4 induced Hepato-toxicity

Hepatotoxicity induced with CCl_4 reformed the levels of hepatic serum bio-markers in the animals, the results in Table 8 represent the levels of ALP different groups, levels of ALP in the Disease Control group is drastically increased compared to the normal levels of ALP in the Normal group. Pre-treatment with Silymarin prevented the elevation of the ALP levels remarkably. Pre-treatment of the fractions also tend to have a control over the increasing levels of ALP in a dose dependent manner. Pre-treatment with 150 mg/Kg body weight had significant effect on the elevated levels and have comparable activity to that of Silymarin standard.

Effect of C2 Column Fraction on Total proteins levels in CCl_4 induced Hepato-toxicity

CCl_4 altered the levels of serum bio-markers in the animals; the results in Table 9 indicate the average SGPT levels in the blood. The levels of total protein are decreased compared to the normal levels of SGPT in the Normal group. Pre-treatment of Silymarin

prevented the decrease of protein levels. Pre-treatment of the Column fractions also showed control over the decreasing levels of proteins in a dose dependent fashion.

Results are expressed as Mean±SEM of Six animals from individual groups, the results were analysed by One way ANOVA followed by Dunnett's comparision-tests. * represents significant at $p<0.05$, ** represents significant at $p<0.01$ and *** represents highly significant at $p<0.001$, all the values were compared with the disease control and were considered as statistically significant.

Elevation in the levels of total bilirubin, conjugated (direct) bilirubin, unconjugated (indirect) bilirubin was observed in CCl_4 induced animals, CCl_4 Altered the levels of serum bio-markers in the animals, the average levels of bilirubin is discussed in Table 9, The levels of Total bilirubin, Direct bilirubin and Indirect bilirubin are increased compared to the normal levels in the Normal group. Pre-treatment of Silymarin prevented the increasing levels of serum bio-markers. Pre-treatment of the Column fractions also showed control over the increasing levels of Total bilirubin, Direct bilirubin and Indirect bilirubin in a dose dependent manner, treatment with 150 mg/Kg had prominent effect on the increasing levels and have comparable activity to that of Silymarin standard.

Histopathological observations

Figure 3 illustrates representative liver sections stained with hematoxylin and eosin from various experimental groups. Sections A, B and C depict the liver tissue of the Normal control, Disease control and Silymarin treatment group, respectively. These sections serve as references for healthy liver tissue, diseased liver tissue and liver tissue under treatment with Silymarin.

Sections D, E and F represent liver sections of animals treated with Column fractions C-2 at different concentrations. These sections provide insight into the effects of Column fractions C-2 on liver tissue morphology at varying doses. Comparing these sections with the control groups allows for an assessment of the potential hepatoprotective effects of Column fractions C-2 and their dose-dependent response.

LCMS analysis

The LCMS analysis produced chromatograms in both positive and negative ionization modes, revealing distinct peaks corresponding to the compounds of interest. Figures 4 and 5 display the Total Ion Chromatogram (TIC) and Base Peak Ion (BPI) chromatograms for the positive and negative ionization modes, respectively. In the positive mode, peaks corresponding to stigmaterin, Kaempferol and quercetin were identified. Similarly, in the negative mode, peaks corresponding to rutin, luteolin and quercetin were observed. The chromatographic separation achieved using the specified gradient program and column conditions facilitated clear identification and quantification of the target compounds. Overall, the LCMS analysis provided comprehensive results, enabling the characterization of the analytes present in the sample.

DISCUSSION

The hepatoprotective potential of natural products has garnered significant interest due to their diverse bioactive constituents and potential therapeutic benefits in liver disorders.[1] The hepatoprotective effect of Cassia auriculata leaf extracts and fractions has been extensively studied. The methanolic extract of Cassia auriculata leaves has been found to possess potent hepatoprotective activity against carbon tetrachloride-induced liver damage in Wistar albino rats. This effect is attributed to the inhibition of hepatic metabolizing enzymes and antioxidant activity, which helps to reduce elevated levels of serum AST, ALT, ALP, total bilirubin, and total cholesterol, while also restoring the abnormal levels of enzymatic antioxidants and MDA in the liver.[51,52] In this study, we aimed to investigate the hepatoprotective activity of the C2 column fraction obtained from *Cassia auriculata* leaves using a CCl_4-induced hepatotoxicity animal model. The comprehensive methodology employed for extraction, fractionation and evaluation of bioactivities provided a robust framework for elucidating the therapeutic potential of the C2 fraction.

The aqueous extract of *Cassia auriculata* (CAE) was meticulously prepared using a systematic approach involving washing, drying, grinding and ultrasound-assisted extraction.[29,30] Liquid-liquid fractionation of the hydroalcoholic extract yielded distinct fractions, with the ethyl acetate fraction (C2) emerging as the focus of further investigation due to its potent antioxidant activity observed in the DPPH assay.

In the DPPH assay, the *C. auriculata* extract and its solvent fractions demonstrated significant antioxidant potential, with the ethyl acetate fraction exhibiting the highest inhibition of DPPH radicals. This finding was further supported by TLC analysis, which revealed the presence of multiple chemical entities in the ethyl acetate fraction,

Subsequently, the ethyl acetate fraction was subjected to *in vitro* and *in vivo* evaluations to assess its hepatoprotective activity. In the *in vitro* cytotoxicity assay using HepG2 cell lines, the ethyl acetate fraction demonstrated dose-dependent protective effects against H_2O_2-induced hepatotoxicity, further highlighting its potential therapeutic utility.

Building upon the promising *in vitro* results, we conducted an in-vivo evaluation of the hepatoprotective activity of the C2 column fraction in a CCl_4-induced hepatotoxicity animal model. The induction of hepatic damage by CCl_4 resulted in alterations in hepatic serum biomarkers, indicative of hepatic dysfunction. Pre-treatment with Silymarin, a standard hepatoprotective agent, effectively mitigated the elevation of hepatic serum biomarkers,

validating the experimental model and serving as a positive control.

Importantly, pre-treatment with the C2 column fraction demonstrated significant hepatoprotective effects in a dose-dependent manner. Administration of the C2 fraction led to dose-dependent reductions in the levels of hepatic serum biomarkers, suggesting its potential therapeutic efficacy in ameliorating CCl_4-induced hepatic damage. Histopathological examination further supported the hepatoprotective effects of the C2 fraction, revealing improvements in liver architecture and attenuation of CCl_4-induced hepatic lesions.

The LCMS analysis provided insights into the chemical composition of the C2 fraction, identifying several bioactive compounds, including quercetin, kaempferol, stigmaterin, rutin, and luteolin. These compounds are known for their antioxidant and hepatoprotective properties and their presence in the C2 fraction further supports its potential therapeutic efficacy.[43,50]

CONCLUSION

In conclusion, this study presents a simplified column fractionation approach guided by DPPH assay to isolate a potent hepatoprotective fraction from *Cassia auriculata*. Through a combination of *in vitro* and *in vivo* experiments, the antioxidant and hepatoprotective properties of *C. auriculata* extract and its fractions were systematically evaluated. The findings underscore the significant potential of *C. auriculata* as a natural therapeutic agent for liver diseases. The observed antioxidant activity and hepatoprotective effects against H_2O_2-induced hepatotoxicity in HepG2 cell lines highlight the promising pharmacological profile of the plant extract. Additionally, the evaluation of the most active Column fraction (C2) against CCl_4-induced hepatotoxicity in albino Wistar rats provides valuable insights into its therapeutic efficacy *in vivo*.

Furthermore, LCMS analysis facilitated the identification of bioactive compounds in the *C. auriculata* extract, including stigmaterin, kaemferol, quercetin, rutin, luteolin and other phytochemicals. These compounds are known for their hepatoprotective and antioxidant properties, contributing to the observed pharmacological effects of the plant extract. The correlation between antioxidant activity and phenolic, tannin and flavonoid contents elucidates the mechanisms underlying the observed pharmacological activities. This study contributes to the growing body of evidence supporting the use of simplified column fractionation guided by DPPH assay for the isolation of potent hepatoprotective fractions from medicinal plants like *C. auriculata*.

Moving forward, future research should focus on exploring the clinical potential of *C. auriculata* extract and its fractions in human subjects. Long-term studies evaluating safety profiles, efficacy in different liver disease models and potential drug interactions are essential for translational applications. Additionally, investigating synergistic interactions between bioactive compounds within the plant extract may lead to the development of novel therapeutic formulations with enhanced efficacy. Overall, this study demonstrates the effectiveness of simplified column fractionation with DPPH assay guidance in isolating potent hepatoprotective fractions, paving the way for the development of new therapeutic interventions for liver diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ANOVA: Analysis of Variance; **CAE:** Cassia auriculata Extract; **CCl4:** Carbon Tetrachloride; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **GAE:** Gallic Acid Equivalents; **H_2O_2:** Hydrogen Peroxide; **HE:** Hematoxylin and Eosin; **IC_{50}:** Half Maximum Inhibitory Concentration; **LCMS:** Liquid Chromatography-Mass Spectrometry; **MTT:** Thiazolyl Blue Tetrazolium Bromide; **SGOT:** Serum Glutamic-Oxaloacetic Transaminase; **SGPT:** Serum Glutamate Pyruvate Transaminase; **TFC:** Total Flavonoid Content; **TLC:** Thin Layer Chromatography; **TPC:** Total Phenolic Content; **TTC:** Total Tannin Content; **UV:** Ultraviolet, **µg/mL:** Micrograms per Milliliter; **HepG2:** Human Liver Cancer Cell Line; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **ALP:** Alkaline Phosphatase; **R_f:** Retardation factor.

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