Chapter 3: Extraction, purification and Characterization of Bioactive constituents from *Hemidesmus indicus* and *Ichnocarpus frutescens*

3.1 Introduction

The search for new therapeutic agents has increasingly focused on natural sources, particularly medicinal plants, which have long been a cornerstone of traditional medicine systems. *Hemidesmus indicus* and *Ichnocarpus frutescens* have broad-spectrum therapeutic properties, and because of these properties, these plants are extensively used in traditional Indian medicine. These plants are rich in secondary metabolites, including flavonoids, saponins, tannins, and alkaloids, which contribute in their therapeutic activities like anti-inflammatory, antidiabetic, antioxidant, and anti-cancer activities (Darshini et al., 2024; Yogeshwari and Kumudha, 2018)

Among the various secondary metabolites, flavonoids are particularly significant due to their potent biological activities. Flavonoids consist of a group of polyphenolic compounds, these are available in large number in the whole plant kingdom and are well-known for their strong antioxidant properties (Karak, 2019). In addition to their antioxidant capabilities, flavonoids also exhibit anti-inflammatory, antidiabetic, and anticancer potentials (Sangeetha et al., 2016). In *Hemidesmus indicus* and *Ichnocarpus frutescens*, flavonoids are considered key contributors to the therapeutic effects of these plants, making them a primary focus of this research.

To further discover the therapeutic properties of flavonoids, it is essential to effectively extract and isolate them from plant materials. One efficient method is the Aqueous Two-Phase System (ATPS), which is used for the separation of flavonoids from complex plant extracts (C. Liu et al., 2018; Zhu et al., 2022). ATPS, a partitioning technique, utilizes two immiscible aqueous solutions, typically composed of a polymer like polyethylene glycol

(PEG) and salt-like ammonium sulfate (Iqbal et al., 2016). This system takes advantage of the differential solubility of compounds, enabling the separation of flavonoids based on their hydrophilic and hydrophobic properties (Buarque et al., 2022). ATPS offers several advantages, including high selectivity, scalability, fast processing, high purity, and high yield. It also has low toxicity and maintains the biological activity of molecules (Bekavac et al., 2024). Additionally, ATPS can handle large sample volumes, making it an ideal method for isolating flavonoids from the crude extracts of *Hemidesmus indicus* and *Ichnocarpus frutescens* (He et al., 2016; Nguyen et al., 2024).

To separate flavonoid from a complex mixture, Silica gel column chromatography is employed widely (Soukup and Jandera, 2012; Sun et al., 2014). In this method, a column packed with silica gel (stationary phase) is used in which flavonoids are passed through it along with a solvent (mobile phase) which leads to the separation of this flavonoid into individual components based on their polarity (Jandera, 2011). Silica gel column chromatography is highly effective in purifying flavonoids due to its capability to handle complex mixtures and separate compounds with similar properties (Janas et al., 2016; Jandera, 2011). This technique ensures the isolation of pure flavonoids, which are crucial for subsequent biological testing and characterization.

Several characterization techniques are commonly used for the characterization and identification of secondary metabolites, including Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Nuclear Magnetic Resonance (NMR) spectroscopy (Hefny Gad et al., 2018). The TLC, a simple technique, provides rapid preliminary information about the presence of flavonoids (de Rijke et al., 2006). HPLC is a more sophisticated method that allows for the precise separation, identification, and quantification of flavonoids (Petrásková et al., 2020). NMR spectroscopy, both 1H-NMR and 13C-NMR, gives insights into the 2D molecular

structure of flavonoids, revealing the arrangement of atoms and functional groups (Hernandes et al., 2020). Additionally, High-Resolution Mass Spectrometry (HR-MS) is used to provide accurate mass measurements, further confirming the identity of the flavonoids (van Dinteren et al., 2021). Therefore, the combined use of TLC, HPLC, NMR, and HRMS enabled the successful identification and confirmation of silychristin and hyperoside in *Hemidesmus indicus* and *Ichnocarpus frutescens*.

3.2 Materials and Methods

3.2.1 Plant material and authentication

Hemidesmus indicus and *Ichnocarpus frutescens* were acquired from Ramna village of Varanasi, Uttar Pradesh, India, in September 2021. *Hemidesmus indicus* and *Ichnocarpus frutescens* were authenticated and assigned by Voucher number "Apocyna. 2022/1" and "Apocyna. 2022/2" by the Head of the Department, Professor N. K. Dubey, Department of Botany, Banaras Hindu University, Varanasi, India and deposited in the herbarium of his department.

3.2.2 Solvent extraction

The roots of both plants, *Hemidesmus indicus* and *Ichnocarpus frutescens* were detached from the whole plant and cleaned with water to get rid of the contaminants. After that, they were shade-dried for 20 days, pulverized into powder, and passed through 20-micrometer mesh-size sieves. Plant extraction was done by the maceration method, in which solvent and root were used in 1:10 ratio. The powdered roots were added into solvent in Erlenmeyer flasks and kept in shaking incubator for 48 hrs at 150 rpm and 40 ◦ C. Solvents like ethyl acetate, methanol, chloroform, and distilled water were employed for extraction. The supernatant liquid was filtered through Whatman filter paper no. 1 to remove debris, and the solvent in the filtrate was evaporated to drying using a rotary evaporator (Nn, 2015). The dried powder extract was dissolved in 10% dimethyl

sulphoxide with 20mg/ml concentration and stored at 4 for further use. In end, the extraction yield was obtained using the following formula (Truong et al., 2019):

$$\text{Extraction yield Percentage} = \frac{\text{Weight of the extract}}{\text{Dry weight of the sample}} \times 100$$

3.2.3 Preliminary qualitative analysis

Preliminary phytochemical analysis of ethyl acetate, chloroform, methanol, and water extracts of root of *Hemidesmus indicus* was ascertained by standard methodologies outlined by (Basiru, A., Ibukun, E., Edobor, G., Ojo, O. and Onikanni, 2013; Ezeonu and Ejikeme, 2016; Usman et al., 2009) to screen presence of flavonoids, tannins, saponin, steroids, terpenoids, alkaloids, glycosides, cardiac glycosides, phenolics, anthraquinone, protein, reducing sugars and carbohydrates.

3.2.3.1 Tannins Test (Ferric chloride method)

A few drops of 0.1 percent ferric chloride in 2.5 mL filtrate produced a brownish-green or blue-black hue, indicating the presence of tannins.

3.2.3.2 Saponin Test

Three drops olive oil was put into the 2.5 ml of solvent extract. After vigorously shaking for 15 min, the foam layer indicates the presence of saponin.

3.2.3.3 Steroids Test (Liebermann-Burchard test)

1ml acetic anhydride was put in 2.5 ml filtrates, followed by 1 ml Sulphuric acid. Violet to blue or green color changes indicated the steroids in the plant.

3.2.3.4 Terpenoids Test (Salkowski Method)

2.5 ml filtrates were taken in the test tube with 1 ml chloroform and 1.5 ml concentrated sulphuric acid. After shaking, the presence of a reddish-brown color at the interface shows the existence of terpenoids.

3.2.3.5 Flavonoids Test (Alkaline reagent Method)

In 5 ml filtrate, 2.5 ml dilute NaOH and 2.5 ml concentrated sulphuric acid was put on. The presence of yellow color specified the presence of flavonoids.

3.2.3.6 Alkaloids Test (Wagner's Method)

1 mL filtrate was placed in a test tube, which was then filled with 5 mL of 1% HCl and boiled in a water bath. 1 ml of this mixture was placed into a test tube after chilling and filtering, and a few drops of Wagner's reagent were added. Alkaloids can be identified by the presence of a reddish-brown precipitate.

3.2.3.7 Glycosides Test

5ml filtrate was measured in a test tube, in which 0.2 ml Fehling solution 1 and 2 were added. After mixing and heating in the water bath, the appearance of brick-red color gives a positive test for glycosides.

3.2.3.8 Cardiac-glycosides Test (Keller-Killani Method)

1 mL glacial acetic acid and a few drops of ferric chloride were added to 2.5 mL filtrate. Following that, 0.5 mL concentrated sulphuric acid was added. The presence of cardiac glycosides is indicated by the formation of a violet-green color ring.

3.2.3.9 Phenolic compounds Test (Ferric chloride Method)

The presence of phenolic compounds is indicated by the emergence of a dark-green color after adding a few drops of 5 percent ferric chloride solution to the 2 ml filtrate.

3.2.3.10 Anthraquinone Test (Borntranger's Method)

A test tube containing 2.5 mL of filtrate was filled with 5 mL of benzene and strained. After that, 2.5 mL of 10% ammonia was added to the filtrate. Pink, crimson, or violet coloration in the ammoniacal phase indicates the presence of anthraquinone.

3.2.3.11 Test for Carbohydrates (Molish test)

2 to 4 drops of 1% alpha-naphthol were placed in 2 ml filtrate, followed by shaking. A violet ring appeared, which marked the occurrence of carbohydrates.

3.2.3.12 Reducing sugar Test (Fehling Method)

1ml each Fehling solution 1 and 2 were added to the test tube containing 2 ml filtrate and boiled in water bath for 5 to 10 minutes. Color change from green to red precipitate denotes the availability of reducing sugars.

3.2.3.13 Protein Test (Biuret Method)

2 mL of 10% NaOH was added to 2 mL filtrate and boiled in a water bath for 10 minutes. Following that, a few drops of 7% copper sulphate were applied. The presence of protein is indicated by the emergence of violet colour.

3.2.4 Quantitative phytochemicals analysis

To assess the total phenolic and flavonoid content, the dried extract obtained from water, methanol, ethyl acetate, and chloroform solvents were dissolved in methanol forming 1mg/ml.

3.2.4.1 Total phenolic content

It was calculated by the Folin-Ciocalteu method, as outlined by Chigurupati et al. (Chigurupati et al., 2021a). A mixture of 1 ml of plant extract and 1 ml of Folin-Ciocalteu was left for 10 minutes. 2 ml of 2.5% Na_2Co_3 was added and kept for 1 hr in the dark condition at room temperature. After incubation, using a UV-Vis Spectrophotometer, the absorbance of all the samples was taken at 765 nm against blank. Gallic acid was taken as a baseline, and its stock solution was prepared in methanol at 1mg/ml concentration. It was expressed as mg gallic acid equivalent (GAE) per gram of extract (extract dry weight).

3.2.4.2 Total flavonoid content

It was assessed by the Aluminium chloride methodology, explained by Chang et al., with minor changes (C. C. Chang et al., 2002). 0.5 ml of 5 percent AlCl3 and 0.5 ml of 1M potassium acetate with 2 ml of each plant solvent extract were incubated in the dark at room temperature for 30 minutes. After that, OD of all the samples was taken at 415 nm by utilizing UV-Vis Spectrophotometer. It was determined as mg quercetin equivalent (QE) per gram extract. Quercetin was the standard its stock solution was prepared in methanol at 1mg/ml concentration.

3.2.5 Elemental Analysis

3.2.5.1 Carbon, Hydrogen and Nitrogen (CHN) Analysis

Quantification and analysis of carbon, hydrogen, and nitrogen in the methanolic root extracts of *Hemidesmus indicus* and *Ichnocarpus frutescens* were performed using a CHNS/O Elemental Analyzer (Thermo Fisher Scientific, FlashSmart V CHNS/O Model). This process is based on the principle of the Dumas method, where flash combustion of the sample occurs, and the resulting products are separated and detected using a chromatographic column and a thermal conductivity detector (TCD), respectively (Szymczycha-Madeja et al., 2013). The built-in chromatographic column converts the compounds, eluting them in the form of NO2, CO2, SO2, and H2O, which are then detected by the TCD. For the analysis, 4-5 mg of the dried methanolic root extract was subjected to combustion at 1800 °C within the analyzer's combustion chamber (Upasana Ghosh, 2013).

3.2.5.2 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

Metal analysis of the root extract of *Hemidesmus indicus* and *Ichnocarpus frutescens* were performed using ICP-AES, using the ARCOS Simultaneous ICP Spectrometer (SPECTRO Analytical Instruments GmbH, Germany). Based on an emission

spectrophotometric approach, ICP-AES detects energy emitted by each element at particular wavelengths that are indicative of its atomic structure. From the upper vacuum ultraviolet region (160 nm) to the edge of visible light (800 nm), the wavelengths utilized in AES (Kamble and Pawar, 2017; Masson et al., 2010).

3.2.6 HR-LCMS

High performance – liquid chromatography-mass spectrometry was utilized to obtain the phytochemical profile of crude methanolic root extract of *Hemidesmus indicus* and *Ichnocarpus frutescens* as the extractive value of methanolic extract was highest. The sample was analyzed using Agilent Technology, USA, having 1290 infinity UHPLC PDA Mass Spectrometry System, 1260 infinity Nano HPLC with chip cube, and 6550 iFunnel Q-TOFs. The column used was Hypersil GOLD C18 100 x 2.1mm-3 MICRON. Secondary metabolites were identified via their unique mass fragmentation patterns and mass spectra (Noumi et al., 2020). Electrospray Ionization Technique provided positive and negative electrospray ionization.

3.2.7 Isolation of Bioactive Constituents

The shade-dried and powdered roots of *Hemidesmus indicus* and *Ichnocarpus frutescens* were collected for extraction. Each extraction began by adding 1 kg of powdered root material to 3 liters of methanol, with the mixture stirred at a speed of 150 rpm and maintained at a temperature of 45°C to enhance the extraction efficiency. After 72 hours of maceration, the extract was filtered to remove solid residues, and the filtrate was concentrated under reduced pressure using a rotary evaporator. To fully extract as many bioactive chemicals as possible, this extraction procedure was carried out three times (Bhanukiran et al., 2023; Nakbanpote et al., 2019). The procedure resulted in 240 g of methanolic extract from *Hemidesmus indicus* and 290 g from *Ichnocarpus frutescens*.

To separate flavonoids from the methanolic extracts, an Aqueous Two-Phase System (ATPS) was employed (Zhang et al., 2013). The ATPS was composed of dipotassium phosphate (K2HPO4), ethanol, water, and the plant extract, mixed in the following proportions: 29% K2HPO4, 25% ethanol, 45% water, and 1% plant extract. This mixture was placed in a separating funnel and stirred for 2 hours at 25°C to facilitate thorough mixing and interaction of the components. After stirring, the mixture was left undisturbed for 5 hours to allow phase separation. This process resulted in the formation of two distinct phases: the upper phase, which contained the flavonoids, and the lower phase, which contained salts and other impurities. The upper liquid phase was carefully collected and dried under reduced pressure using a rotary evaporator to obtain a concentrated extract of flavonoids as shown in Figure 3.1 (He et al., 2016; Ma et al., 2013).

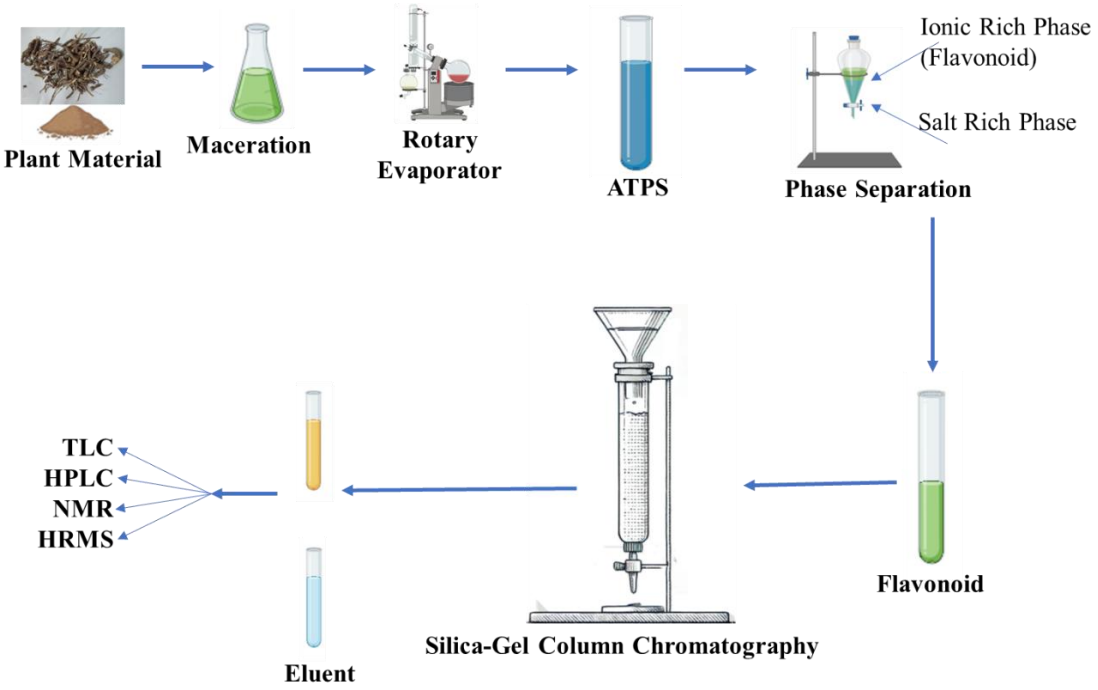

Figure 3.1: Process of Isolation of Silychristin from *Hemidesmus indicus* and Hyperoside from *Ichnocarpus frutescens*

Following the phase separation, the bioactive compounds were isolated using the silica gel column chromatography technique (He et al., 2014). The column was packed with silica gel (60-120 mesh) by preparing a slurry in hexane, which was then carefully poured

into the column while tapping it to ensure uniform packing without breaks or air gaps in the silica bed. The flavonoid, 32 g from *Hemidesmus indicus* and 26g from *Ichnocarpus frutescens*, were loaded onto the different columns. A gradient elution method was used for the chromatographic separation. The process began with a non-polar solvent, 100% hexane, to elute less polar compounds. The polarity was then gradually increased by introducing solvents of higher polarity, first ethyl acetate, followed by dichloromethane, and finally a mixture of 10% methanol. This stepwise increase in solvent polarity facilitated the separation of flavonoids based on their differing affinities to the stationary and mobile phases (Bhanukiran et al., 2023).

The eluents collected from the column were transferred into flasks, and each fraction was analyzed using TLC. Silica gel 60 F254-precoated TLC plates were utilized to detect flavonoid-containing fractions. The mobile phase for TLC consisted of a mixture of ethyl acetate, methanol, formic acid, and a few drops of water in a ratio of 7:2:1:0.1, respectively. To identify flavonoid components, the produced TLC plates were examined in an iodine chamber and under UV illumination at 254 nm. Fractions showing a single spot with the same retention factor (Rf value) were indicative of the presence of pure compounds and were pooled together. The pooled fractions were subjected to structural elucidation to confirm the identity of the flavonoids (Kabamba et al., 2019; Muhammad et al., 2015).

3.2.8 Characterization of Isolated Compounds

The isolated compounds, silychristin from *Hemidesmus indicus* and hyperoside from *Ichnocarpus frutescens*, were characterized using several analytical techniques: TLC, HPLC, NMR, and Mass Spectrometry (MS).

3.2.8.1 Thin-Layer Chromatography (TLC)

TLC was performed on the isolated fractions containing silychristin from *Hemidesmus indicus* and hyperoside from *Ichnocarpus frutescens*. The mobile phase used was a mixture of ethyl acetate, methanol, formic acid, and water in the ratio of 7:2:1:0.1 (Kabamba et al., 2019). The stationary phase comprised Silica Gel 60 F254 plates. Pure silychristin and hyperoside were used as standard compounds for comparison. The following formula was used to ascertain the retention factor (Rf) of isolated compounds:

$$\text{Retention factor (Rf)} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent front}}$$

This method allowed for the identification of silychristin and hyperoside by comparing their Rf values to those of the standard compounds (Banso et al., 2024).

3.2.8.2 HPLC Analysis

HPLC analysis was conducted to further detect and confirm the presence of silychristin and hyperoside in the fractions isolated from *Hemidesmus indicus* and *Ichnocarpus frutescens*. The analysis was performed using a Shimadzu LC-20AD system equipped with dual binary pumps and a Shimadzu Prominence SPD-M20A PDA detector. A C-18 reversed-phase column (Phenomenex, Gemini 5 µm, 250 mm length × 4.6 mm internal diameter) was used for separation. Two solvents were used: solvent A (water with 0.5% trifluoroacetic acid) and solvent B (acetonitrile with 0.5% trifluoroacetic acid), mixed in a 60:40 ratio (Inbaraj et al., 2010). These solvents were filtered through a 0.45 µm membrane filter and degassed using an ultrasonic method before use. The samples were prepared in methanol and also filtered through a 0.45 µm membrane. A 20 µl sample injection volume was used, with the pump flow rate set at 0.8 ml/min, and the total run time was 10 minutes. Silychristin was detected at a wavelength of 288 nm, while hyperoside was detected at 340 nm (Cheng et al., 2007; Liu et al., 2010; Shen et al., 2023;

Wang et al., 2010). Comparative analysis of the retention times and peak areas of the isolated fractions with those of pure silychristin and hyperoside standards confirmed their presence.

3.2.8.3 NMR Analysis

NMR spectroscopy was performed to elucidate the structural details of the isolated compounds. Both 1H NMR and 13C NMR analyses were carried out using a Bruker Avance Neo 600 MHz NMR spectrometer. Tetramethylsilane (TMS) was used as the internal standard, and CD3OD (deuterated methanol) served as the solvent for sample preparation. These analyses provided detailed information about the hydrogen and carbon environments within the silychristin and hyperoside molecules (Mal et al., 2021; Tsolis et al., 2024).

3.2.8.4 Mass Spectrometry Analysis

High-resolution mass spectrometry (HRMS) was performed to ascertain the molecular masses of silychristin and hyperoside. These studies were performed using a SCIEX X500R QTOF mass spectrometer. The HRMS analysis provided accurate molecular weights, further confirming the identity and purity of the isolated compounds. Together, these techniques ensured the accurate characterization and confirmation of the bioactive compounds silychristin and hyperoside, isolated from *Hemidesmus indicus* and *Ichnocarpus frutescens*, respectively (Massano et al., 2023; Salomone et al., 2021).

3.3 Results and Discussions

3.3.1 Extraction Yield Qualitative Analysis

Plant extractive yield depends on several factors, including the type of solvent, solvent polarity, extraction method, temperature, plant parts used, extraction time, and the size and composition of the samples (Do et al., 2014a). Since many phytochemicals are polar, extractive yield generally increases with the polarity of the solvent. Solvents that produce

higher extractive yields are expected to extract phytoconstituents more effectively. Polar solvents like methanol are particularly effective at extracting polar compounds, such as phenolic compounds, flavonoids, and glycosides, due to their ability to form hydrogen bonds and interact with these compounds (Stalikas, 2007).

In this study, the maceration method was used to determine the extractive yield of *Hemidesmus indicus* and *Ichnocarpus frutescens* roots, employing four solvents with increasing polarities: chloroform, ethyl acetate, water, and methanol. For *Hemidesmus indicus*, methanol yielded the highest extractive yield at 24.70 ± 0.08%, followed by water at 19.2 ± 0.32%, ethyl acetate at 15.73 ± 0.24%, and chloroform at 8.6 ± 0.16%. Similarly, for *Ichnocarpus frutescens*, methanol produced the highest extractive yield at 28.73 ± 0.89%, followed by water at 20.09 ± 1.05%, ethyl acetate at 14.13 ± 0.18%, and chloroform at 9.53 ± 1.35%. The extractive yield of both plants has been depicted in Figure 3.2**.**

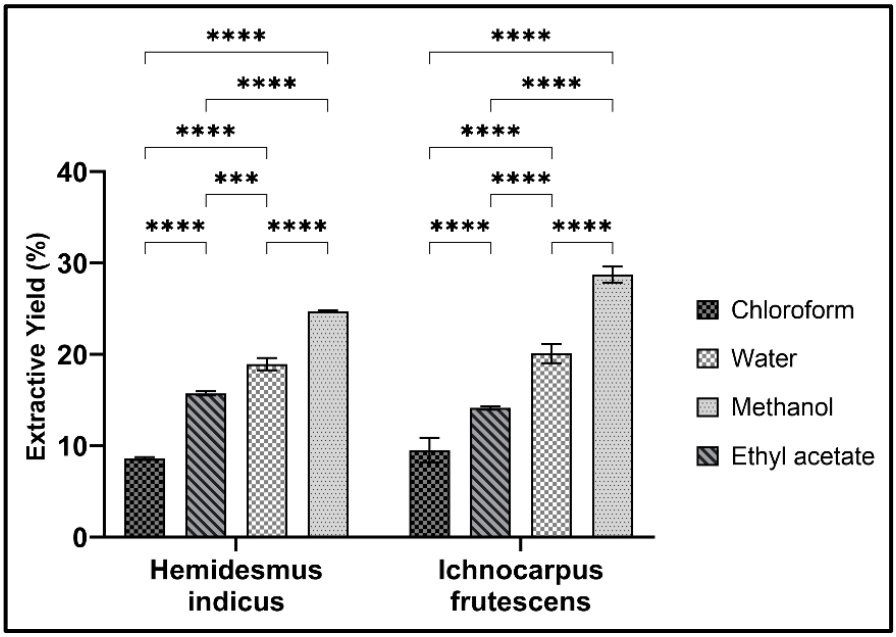

Figure 3.2: Extractive Yield of *Hemidesmus indicus* and *Ichnocarpus frutescens* root in different solvents. Bars show mean±SD; n= 3 independent extractions per solvent per species. Statistics were performed in GraphPad Prism using a two-way ANOVA (factors: species × solvent) followed by Tukey's multiple-comparisons post hoc test. Brackets indicate the pairwise comparisons tested. Significance: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***), $P<0.0001$ (****); ns= not significant ($P= NS$) (two-tailed, $\alpha= 0.05$).

These results indicate that the extractive yield of both plants increases with the polarity of the solvent. This suggests that the plants contain more polar phytochemicals, which are more soluble in highly polar solvents such as methanol and water, compared to less polar solvents like chloroform. The findings are consistent with those of Truong et al. who reported that methanol produced the highest extract yield from the medicinal plant *Severinia buxifolia*, followed by distilled water and chloroform (Truong et al., 2019). This research aligns with other studies on medicinal plants, which have shown that polar solvents are more effective at extracting polar phytochemicals (Anokwuru et al., 2011).

3.3.2 Qualitative Analysis

Qualitative analysis revealed the presence of various secondary metabolites, including carbohydrates, reducing sugars, proteins, flavonoids, alkaloids, glycosides, terpenoids, phenolics, saponins, steroids, anthraquinone, and tannins, in the roots of *Hemidesmus indicus* and *Ichnocarpus frutescens*, as shown in Table 3.1.

Table 3.1: Qualitative phytochemical analysis of *Hemidesmus indicus* and *Ichnocarpus frutescens* in different solvents.

Class of Phytoconstituents	Chloroform		Methanol		Ethyl acetate		Water	
	HI	IF	HI	IF	HI	IF	HI	IF
Tannins	-	+	++	+	-	-	+	+
Flavonoids	-	+	+++	++	+	+	-	+
Phenolics	+	+	+++	+++	++	+	+	++
Terpenoids	+	-	+++	++	++	-	+	++
Steroids	-	+	+	++	-	+	-	-
Alkaloids	+	-	++	+	+	-	+	+
Saponin	+	-	+	++	-	+	-	-
Reducing sugar	+	-	++	+	-	+	+	+
Carbohydrates	+	-	+	++	+	-	+	++
Protein	-	+	+	++	+	+	+	+

These secondary metabolites are known to have a wide range of biological and therapeutic effects, suggesting that the roots of *Hemidesmus indicus* and *Ichnocarpus frutescens* may have numerous therapeutic applications (Benedec et al., 2013; Narender Prasad et al., 2012).

For both plants, methanol was found to extract the highest concentration (+++) of most classes of phytochemicals, followed by water extracts. In contrast, solvents with lower polarity, such as chloroform and ethyl acetate, showed lower extraction efficiency for these phytoconstituents. In the case of *Hemidesmus indicus*, alkaloids, phenolics, and carbohydrates were detected in extracts from all four solvents. However, steroids were found only in the methanol extract, and anthraquinone was present only in the water extract. For *Ichnocarpus frutescens,* flavonoids and phenolic compounds were detected in extracts from all solvents, while anthraquinone was found exclusively in the methanol extract. Glycosides and cardiac glycosides were present in all solvent extracts except for the water extract. These results highlight the importance of solvent choice in extracting specific phytochemicals, demonstrating that more polar solvents like methanol are more effective for extracting a broader range of bioactive compounds from *Hemidesmus indicus* and *Ichnocarpus frutescens.*

3.3.3 Quantitative phytochemicals analysis

The total phenolic content (TPC) and total flavonoid content (TFC) of the roots of *Hemidesmus indicus* and *Ichnocarpus frutescens* were evaluated using chloroform, ethyl acetate, water, and methanol extract, as shown in Figure 3.3 and 3**.4.** These evaluations were conducted to identify the most effective solvent for extracting bioactive compounds. For *Hemidesmus indicus*, the TPC varied from 43.58 mg GAE/g to 154.15 mg GAE/g, while the TFC ranged from 38.6 mg QE/g to 70.61 mg QE/g. Among the solvents tested, methanol showed the highest TPC (154.15 ± 1.24 mg GAE/g) and TFC (70.61 ± 0.35 mg QE/g), indicating its superiority in extracting phenolic and flavonoid compounds. In the case of *Ichnocarpus frutescens*, the TPC ranged from 37.45 mg GAE/g for the ethyl acetate extract to 137.00 mg GAE/g for the methanol extract. The TPC values followed the order: methanol > water > chloroform > ethyl acetate. Similarly, the TFC ranged from

19.92 mg QE/g to 52.59 mg QE/g, with the methanol extract showing the highest flavonoid content, followed by water, ethyl acetate, and chloroform. The higher TPC and TFC in methanol extracts are due to methanol's ability to dissolve phenolic and flavonoid compounds more effectively (Do et al., 2014a). This is because methanol can form hydrogen bonds with polyphenols, enhancing their extraction (Felhi et al., 2017). Additionally, the solubility of phytochemicals is influenced by their level of polymerization and their interactions with each other (Naczk and Shahidi, 2004).

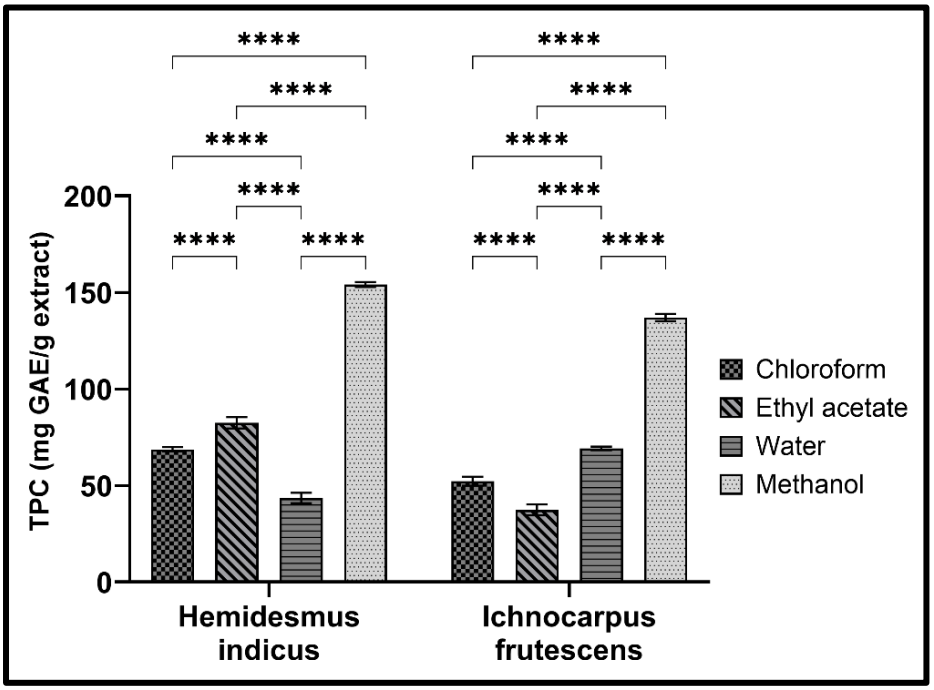

Figure 3.3: Total Phenolic Content (TPC) of *Hemidesmus indicus* and *Ichnocarpus frutescens* root in different solvent extracts. Bars show mean ± SD; n = 3 independent extractions per solvent per species. Statistics were performed in GraphPad Prism using a two-way ANOVA (factors: species × solvent) followed by Tukey's multiple-comparisons post hoc test. Brackets indicate the pairwise comparisons tested. Significance: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****); ns = not significant ($P = NS$) (two-tailed, $\alpha = 0.05$).

Overall, these findings demonstrate that methanol is the most effective solvent for extracting phenolic and flavonoid compounds from both *Hemidesmus indicus* and *Ichnocarpus frutescens*. Therefore, the methanolic extracts were selected for further analysis to identify specific phytoconstituents. These findings emphasize the importance

of selecting appropriate solvents to maximize the extraction of bioactive compounds with potential therapeutic applications.

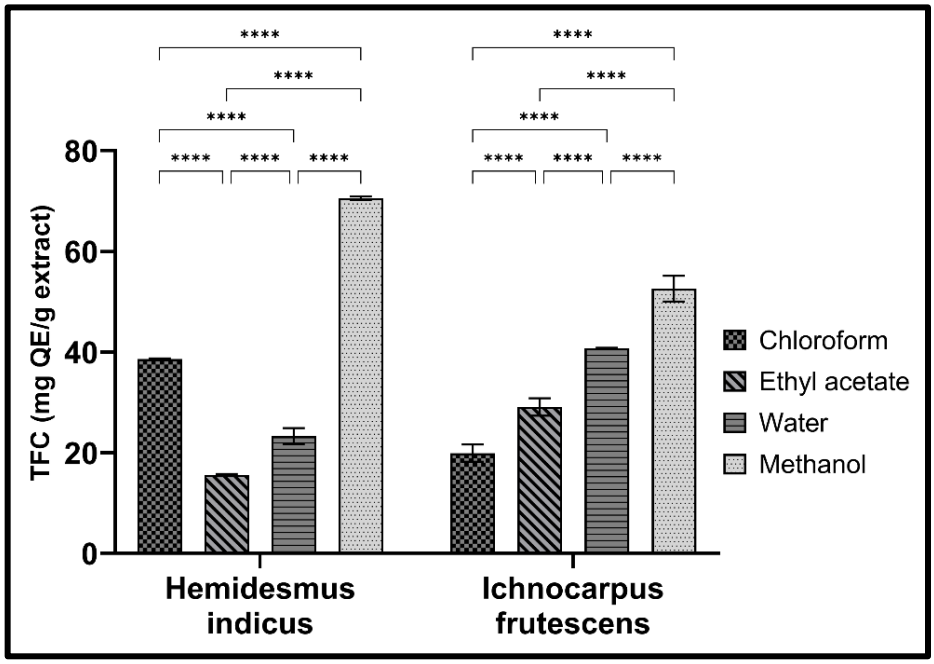

Figure 3.4: Total Flavonoid Content (TFC) of *Hemidesmus indicus* and *Ichnocarpus frutescens* root in different solvent extracts. Bars show mean ± SD; n = 3 independent extractions per solvent per species. Statistics were performed in GraphPad Prism using a two-way ANOVA (factors: species × solvent) followed by Tukey's multiple-comparisons post hoc test. Brackets indicate the pairwise comparisons tested. Significance: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****); ns = not significant ($P = NS$) (two-tailed, $\alpha = 0.05$).

3.3.4 Carbon, Hydrogen and Nitrogen (CHN) analysis

The CHN (Carbon, Hydrogen, Nitrogen) elemental analysis of the methanolic root extracts of *Hemidesmus indicus* (HI) and *Ichnocarpus frutescens* (IF) is depicted in the figure 3.5. This analysis provides the percentage composition of carbon, hydrogen, and nitrogen elements, along with their corresponding retention times. For nitrogen, *Hemidesmus indicus* has a higher nitrogen content of 0.969% compared to 0.181% in *Ichnocarpus frutescens*. The retention times are similar, with HI at 0.767 minutes and IF at 0.775 minutes, indicating that the nitrogen-containing compounds in both extracts have similar chemical properties. However, HI has a significantly greater nitrogen presence, suggesting a higher concentration of nitrogenous compounds.

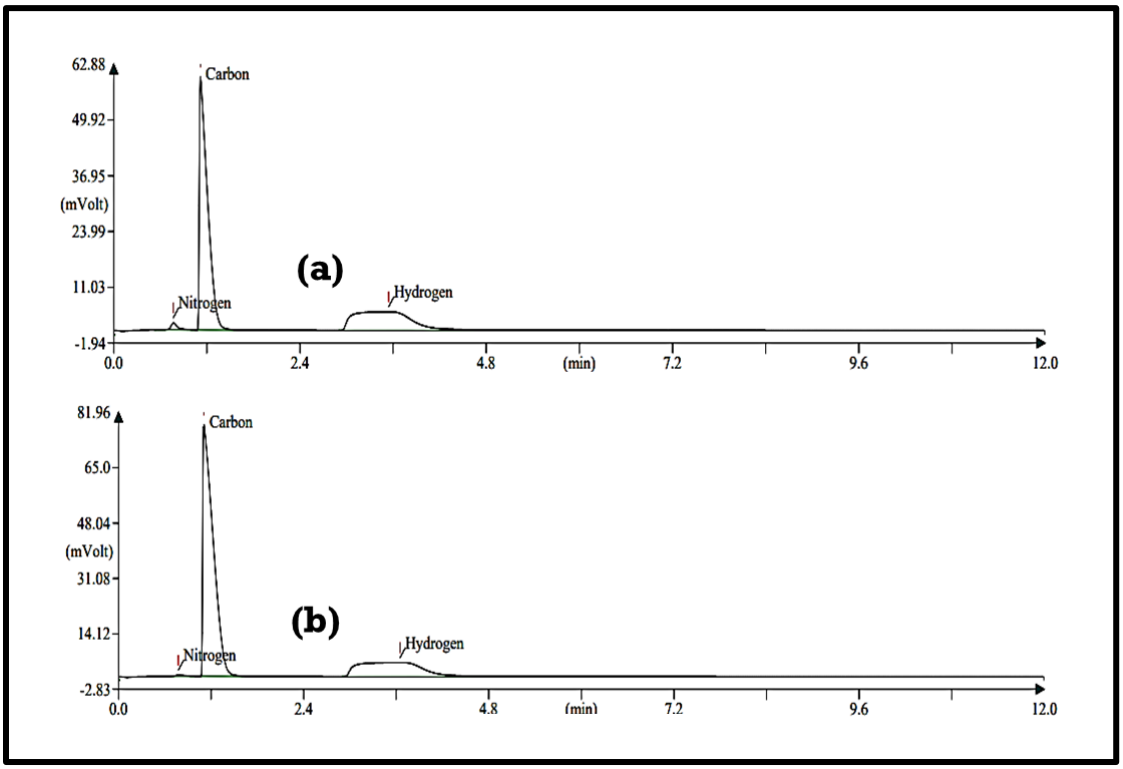

Figure 3.5: CHN elemental analysis of methanolic root extract of (a) representing *Hemidesmus indicus* and (b) representing *Ichnocarpus frutescens.*

Regarding carbon content, *Ichnocarpus frutescens* shows a notably higher carbon percentage at 51.832% compared to 37.758% in *Hemidesmus indicus*. The retention times for carbon are also close, with HI at 1.117 minutes and IF at 1.108 minutes. This higher carbon content in IF suggests a greater presence of carbon-rich compounds, possibly indicating a different composition of organic compounds that contribute to its overall chemical profile.

For hydrogen, both plants have similar contents, with *Hemidesmus indicus* at 6.938% and *Ichnocarpus frutescens* at 6.365%. The retention times are also very close, with HI at 3.542 minutes and IF at 3.650 minutes. This similarity indicates that the hydrogen-containing compounds in both extracts share similar chemical environments, with only a slight difference in concentration. Thus, the CHN elemental analysis reveals that *Hemidesmus indicus* has higher nitrogen content, whereas *Ichnocarpus frutescens* has a higher carbon content. Both plants have similar hydrogen content.

Higher N_2 content in *H. indicus* suggests a greater relative abundance of nitrogen-containing compounds such as alkaloids, amino acids, and nitrogenous flavonoids. These compounds play roles in enzymatic regulation, cell signaling, and interactions with DNA and proteins, which are critical in managing diabetes and cancer (Kumar and Pandey, 2013; Roy, 2017; Zhao et al., 2021). In contrast, a higher carbon content in *Ichnocarpus frutescens* may reflect a greater relative proportion of carbon-rich compounds, such as phenolics, flavonoids, lignans, triterpenes, and other secondary metabolites primarily composed of carbon skeletons. These compounds are typically derived from the phenylpropanoid and polyketide biosynthetic pathways and are key contributors to antioxidant, anticancer, and anti-inflammatory activities (Nowak et al., 2014; Sultana et al., 2009). The similar hydrogen levels in both roots suggest that the overall saturation and oxidation states of the major metabolites are comparable, likely reflecting similar classes of partially reduced organic compounds found in both plant species (Onwudili et al., 2024).

3.3.5 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

The elemental analysis of the roots of *Ichnocarpus frutescens* and *Hemidesmus indicus* was conducted using ICP-AES) a technique that identifies and quantifies the presence of metal elements in samples. For the root of *Ichnocarpus frutescens*, a variety of elements were identified, including Boron (B), Aluminum (Al), Silicon (Si), Phosphorus (P), Sulfur (S), Calcium (Ca), Potassium (K), Sodium (Na), Magnesium (Mg), Zinc (Zn), Iron (Fe), Copper (Cu), and Manganese (Mn). These elements play crucial roles in plant growth, development, and physiological functions, indicating that *Ichnocarpus frutescens* possesses a broad range of essential and trace elements. In the case of *Hemidesmus indicus*, the root was found to contain Lithium (Li), Sodium (Na), Potassium (K), Magnesium (Mg), Calcium (Ca), Aluminum (Al), Iron (Fe), Manganese (Mn), Copper

(Cu), Zinc (Zn), and Sulfur (S). The presence of these elements highlights their importance in the metabolic processes and structural integrity of the plant. Notably, *Hemidesmus indicus* includes Lithium (Li), which is absent in *Ichnocarpus frutescens*.

Both plants contain abundant essential elements such as Ca, Mg, K, Na, Fe, Zn, Cu, and Mn, which are vital for plant metabolism and serve key roles in enzyme activation and antioxidant defense (Broadley et al., 2007; Ju et al., 2017). In medicinal use, they support enzymatic functions and act as cofactors in redox reactions, contributing to antioxidant, antidiabetic, and cytoprotective effects (Pandey and Rizvi, 2009; Sultana et al., 2009). Additionally, these minerals play key roles in phenolic biosynthesis and other metabolic pathways that enhance both plant health and therapeutic efficacy (Cheynier et al., 2013; Jan et al., 2021). The unique presence of boron, phosphorus, and silicon in *Ichnocarpus frutescens* enhances specific therapeutic actions. Boron is associated with anti-inflammatory and metabolic regulation (Herrera-Rodríguez et al., 2010), phosphorus is central to energy transfer (ATP), and silicon supports antioxidant capacity and cell wall resilience, which could provide additional tissue-repairing or adaptogenic effects (Epstein, 1999). In contrast, the detection of lithium specifically in *Hemidesmus indicus* is notable, as lithium is known for neuroprotective and mood-stabilizing effects at trace levels, suggesting unique potential uses for neurological modulation (Malhi et al., 2013). These variations in elemental composition could influence each plant's potential therapeutic properties and applications.

3.3.6 HR-LCMS

HR-LCMS was conducted to identify the phytochemicals present in the methanolic root extracts of Hemidesmus indicus and Ichnocarpus frutescens. Phytochemicals were identified based on various factors, including database matches, retention times, MS/MS fragment patterns, experimental m/z values, metabolite classes, and the number of hits.

Chromatograms for both positive and negative electrospray ionization modes for Hemidesmus indicus and Ichnocarpus frutescens are shown in Figure 3.6 and 3.7.

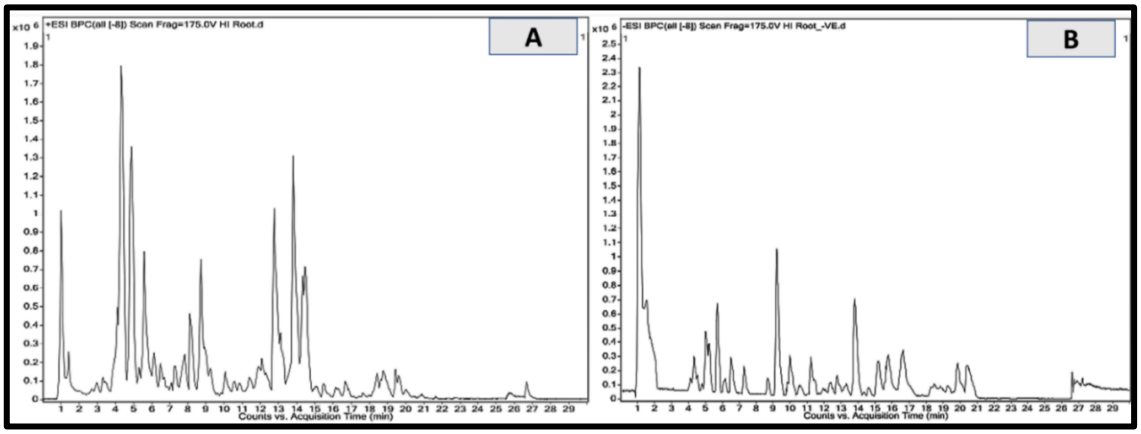

Figure 3.6: HR-LCMS Chromatogram of *Hemidesmus indicus* methanolic extract showing (A) positive and (B) negative electrospray ionization spectra.

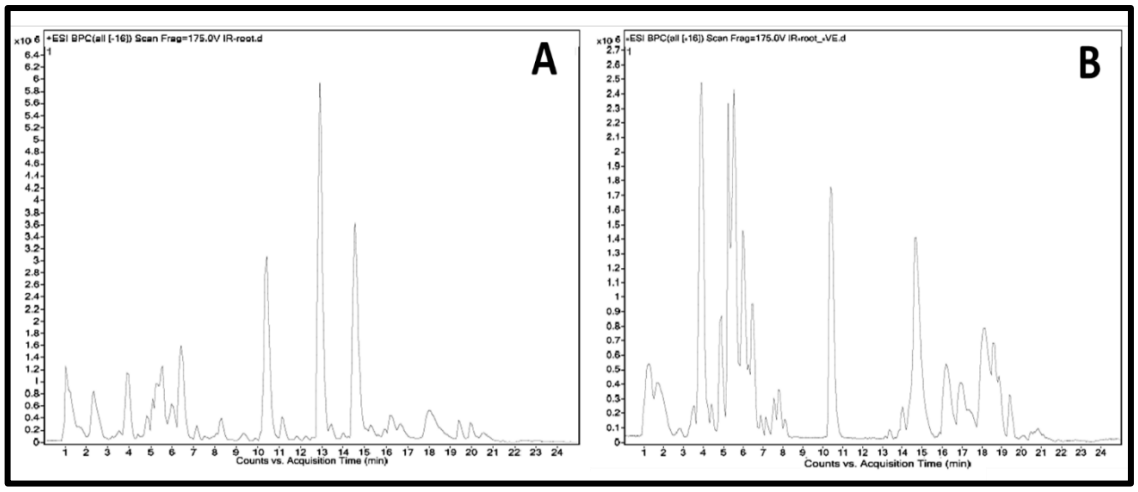

Figure 3.7: HR-LCMS Chromatogram of *Ichnocarpus frutescens* methanol extract showing (A) positive and (B) negative electrospray ionization spectra.

These chromatograms illustrate the use of positive ionization, which forms positive ions [M+H]+ (m/z), and negative ionization, which forms negative ions [M-H]− (m/z), to determine the mass-to-charge (m/z) ratios of the samples (Large and Knof, 1976). For Hemidesmus indicus, the majority of the identified phytochemicals had m/z values ranging from 174 to 693. In contrast, the bioactive compounds from Ichnocarpus frutescens exhibited m/z ratios ranging from 175 to 1131. The phytochemicals were

characterized and identified by comparing the high-resolution liquid chromatography and mass spectra of the plant constituents with a comprehensive library database. The identified phytochemicals from Hemidesmus indicus and Ichnocarpus frutescens are detailed in Tables 3.2 and 3.3, respectively.

Table 3.2: List of secondary metabolites of *H. indicus* from methanolic root extract.

S. N	Phytochemicals	Gp of Phytochemicals	Formula	Retention Time [min]	$[M+H]^+$ (m/z)	$[M+H]^-$ (m/z)
1	Solanocapsine	alkaloid	$C_{27}H_{46}N_2O_2$	10.038	453.3422	-
2	Cyclovirobuxine C	alkaloid	$C_{27}H_{48}N_2O$	13.048	439.3631	-
3	Lucidine B	alkaloid	$C_{30}H_{49}N_3O$	13.288	490.3875	-
4	Zygadenine	alkaloid	$C_{27}H_{43}NO_7$	15.454	-	551.3167
5	Fumitremorgin C	alkaloid	$C_{22}H_{25}N_3O_3$	2.917	402.1751	-
6	Aspidospermidine	alkaloid	$C_{19}H_{26}N_2$	5.581	283.2125	-
7	Ammothamnine	alkaloid	$C_{15}H_{24}N_2O_2$	19.112	-	309.1806
8	Silychristin	flavonoid	$C_{25}H_{22}O_{10}$	4.471	-	481.1168
9	3,3',5-Trihydroxy-4',7-dimethoxyflavanone	flavonoid	$C_{17}H_{16}O_7$	1.327	-	377.088
10	4,5-Di-O-caffeoylquinic acid	Phenolic Compound	$C_{25}H_{24}O_{12}$	6.096	-	515.1273
11	6-Deoxocathasterone	steroid	$C_{28}H_{50}O_2$	18.444	441.3712	-
12	Dolicholide	brassinosteroid	$C_{28}H_{46}O_6$	11.321	-	523.3314
13	ergostan-3β-ol	steroid	$C_{28}H_{50}O$	18.95	425.3763	-
14	Castasterone	steroid	$C_{28}H_{48}O_5$	13.324	-	509.3504
15	(24R)-5alpha-ergostan-3-one	steroid	$C_{28}H_{48}O$	19.444	423.361	-
16	22,23-Dihydroergosterol	steroid	$C_{28}H_{46}O$	20.052	421.3453	-
17	3beta-3-Hydroxy-18-lupen-21-one	triterpenoid.	$C_{30}H_{48}O_2$	18.783	441.3715	-
18	Manglupenone	triterpenoid.	$C_{30}H44O_2$	12.348	437.3474	-
19	17beta-Hydroxyestr-5(10)-en-3-one	steroid	$C_{18}H_{26}O_2$	13.69	275.2001	-
20	19-Noretiocholanolone	steroid	$C_{18}H_{28}O_2$	13.967	277.2155	-
21	Rutaevin	steroid	$C_{26}H_{30}O_9$	4.977	-	485.1852
22	4,5-Di-O-caffeoylquinic acid	phenolic compounds	$C_{25}H_{24}O_{12}$	6.096	-	515.1273
23	5,6,2'-Trimethoxyflavone	flavonoids	$C_{18}H_{16}O_5$	7.359	313.1064	-
24	Plumieride	glycoside	$C_{21}H_{26}O_{12}$	4.334	-	515.1455
25	Fraxetin	hydroxycoumarin	$C_{10}H_8O_5$	6.466	209.0439	-
26	Lucuminic acid	glycoside	$C_{19}H_{26}O_{12}$	4.507	469.1306	-
27	Phlegmarine	glycoside	$C_{16}H_{30}N_2$	13.768	-	295.2337
28	Hordatine B	Phenolic compounds	$C_{29}H_{40}N_8O_5$	14.051	-	639.329

Table 3.3: Secondary metabolites of *Ichnocarpus frutescens* methanolic root extract using HR-LCMS technique.

S. N	Identified Phytochemicals	Class of Phytochemical	Formula	Retention Time [min]	$[M+H]^+$ (m/z)	$[M+H]^-$ (m/z)
1	Lucuminic acid	Glycoside	$C_{19}H_{26}O_{12}$	5.123	469.12	-
2	Atrovirinone	Alkaloids	$C_{25}H_{28}O_8$	3.492	479.17	-
3	Indoleacrylic acid	monocarboxylic acid	$C_{11}H_9NO_2$	3.786	188.06	-
4	23-Acetoxysoladulcidine	Alkaloids	$C_{29}H_{47}NO_4$	16.08	496.33	-
5	Bismurrayaquinone A	Alkaloid	$C_{26}H_{16}N_2O_4$	4.34	-	479.12
6	Asterosterol	Steroid	$C_{26}H_{42}O$	12.89	371.32	-
7	Gambiriin A3	Flavonoid catechin	$C_{30}H_{28}O_{12}$	4.833	-	625.15
8	Lamprolobine	Alkaloids	$C_{15}H_{24}N_2O_2$	16.44	-	309.17
9	Ergostan-3β-ol	Steroid	$C_{28}H_{50}O$	15.27	425.37	-
10	Blumeatin B	Flavonoids	$C_{17}H_{16}O_7$	1.088	-	377.08
11	Fumitremorgin C	Alkaloid	$C_{22}H_{25}N_3O_3$	5.04	-	378.18
12	Zygadenine	Alkaloid	$C_{27}H_{43}NO_7$	5.92	494.30	-
13	Isoplumbagin	Alkaloid	$C_{11}H_8O_3$	6.421	189.05	-
14	Myricetin 7-rhamnoside	Phenolic	$C_{21}H_{20}O_{12}$	5.345	487.08	-
15	Hyperoside	flavonoid	$C_{21}H_{20}O_{12}$	6.74	-	463.09
16	Licogroside B	dicarboxylic acid monoester	$C_{18}H_{24}O_{12}$	3.89	455.11	-
17	Lawsone	quinone	$C_{10}H_6O_3$	3.894	175.03	-
18	Beta-Mangostin	xanthones	$C_{25}H_{28}O_6$	4.99	447.18	-
19	1-(2-Hydroxyphenylamino)-1-deoxy-beta-D-gentiobioside 1,2-carbamate	glycoside	$C_{19}H_{25}NO_{12}$	5.26	482.12	-
20	Dhurrin 6'-glucoside	cyanogenic glucoside	$C_{20}H_{27}NO_{12}$	6.311	496.14	-
21	Decursinol	coumarins	$C_{14}H_{14}O_4$	10.59	269.079	-
22	Aescin	triterpenoid saponin	$C_{55}H_{86}O_{24}$	8.168	1131.55	-
23	Asperuloside	monoterpenoid	$C_{18}H_{22}O_{11}$	1.514	-	413.11
24	Zopiclone	monochloropyridine	$C_{17}H_{17}ClN_6O_3$	4.109	-	447.12
25	Catechin-(4alpha->8)-gallocatechin-(4alpha->8)-catechin	flavonoid	$C_{45}H_{38}O_{19}$	5.27	-	927.19
26	Remikiren	Carboxamide	$C_{33}H_{50}N_4O_6S$	14.00	-	689.36
27	Spinoside A	glycoside	$C_{39}H_{56}O_{12}$	14.46	-	715.37
28	Hovenine A	oligopeptide	$C_{27}H_{42}N_4O_4$	15.92	-	531.32
29	Muzanzagenin	oxo steroid	$C_{27}H_{38}O_5$	17.69	-	441.25

3.3.7 Chemical and structural properties of isolated compounds

To isolate specific phytoconstituents from Hemidesmus indicus and Ichnocarpus frutescens, the silica gel column chromatography technique was utilized. The roots of Hemidesmus indicus and Ichnocarpus frutescens yielded 24% w/w and 29% w/w of methanolic extract, respectively. Using the Aqueous Two-Phase System (ATPS), the flavonoid yield from the methanolic root extract of Hemidesmus indicus was determined to be 13.54% w/w, while for Ichnocarpus frutescens, it was 9.23% w/w. This higher yield of flavonoids from Hemidesmus indicus indicates a greater abundance of these bioactive compounds in its roots compared to those of Ichnocarpus frutescens. To isolate specific phytoconstituents, the flavonoid-rich extracts were subjected to fractionation using silica gel column chromatography. This process resulted in the collection of 20 fractions from each plant extract.

Among these fractions, two significant phytoconstituents were identified. From Hemidesmus indicus, silychristin was isolated, eluting from the fractions number 4-9. In contrast, hyperoside was isolated from Ichnocarpus frutescens, eluting from fractions number 13-17. The percentage yields of the isolated compounds were 0.21% w/w for silychristin and 0.37% w/w for hyperoside. Silychristin (Figure 3.8.A) was characterized as a solid with a whitish colour and a distinctive odour. Hyperoside (Figure 3.8.B.) was identified as a solid with a yellow colour and characteristic odour.

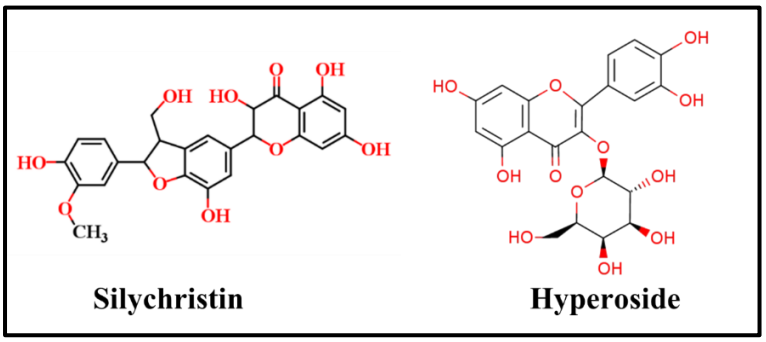

Figure 3.8: The structure of phytochemicals isolated from Hemidesmus indicus (Fig. A. Silychristin) and Ichnocarpus frutescens (Fig. B. Hyperoside)

3.3.7.1 Thin-Layer Chromatography (TLC)

TLC was performed on the isolated fractions from *Hemidesmus indicus* and *Ichnocarpus frutescens* to verify the presence of specific phytoconstituents. The TLC plates were developed using a solvent system consisting of ethyl acetate, methanol, formic acid, and water. Figure 3.9. A show the TLC results for the fractions obtained from *Ichnocarpus frutescens*. Two spots are visible: one corresponding to the standard hyperoside and the other to the fraction from *Ichnocarpus frutescens*. Both spots align at the same level, indicating that they have the same retention factor (Rf). The Rf value for both the standard hyperoside and the fraction from *Ichnocarpus frutescens* was calculated to be 0.62. This matching Rf value confirms that the compound isolated from *Ichnocarpus frutescens* is hyperoside. Figure 3.9.B depicts the TLC results for the fractions obtained from *Hemidesmus indicus*, in which two spots are observed: one corresponding to the standard silychristin and the other to the fraction from *Hemidesmus indicus*.

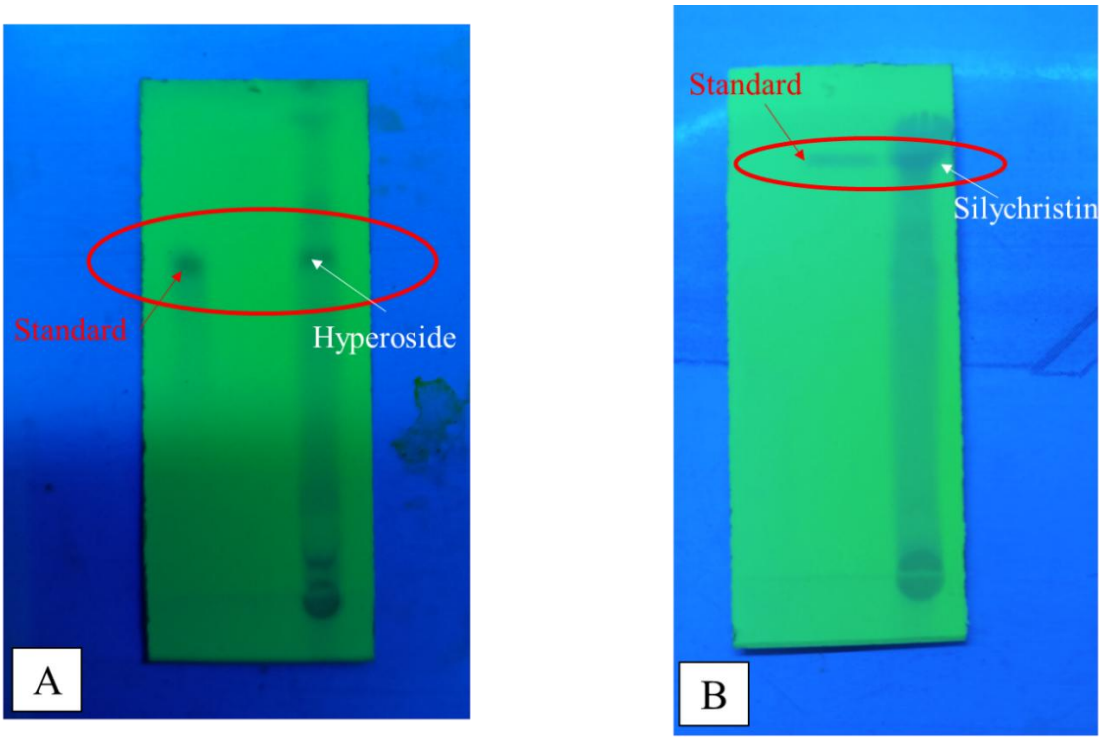

Figure 3.9: TLC image of *Ichnocarpus frutescens* (fig. A) and *Hemidesmus indicus* (fig. B). The standard is respective pure compounds for a reference run.

Both spots have the same Rf value, which was calculated to be 0.97, indicating that the fraction from *Hemidesmus indicus* is identical to the standard silychristin. This confirms the presence of silychristin in the *Hemidesmus indicus* fraction.

3.3.7.2 HPLC Analysis

HPLC was employed to further confirm the presence of the phytoconstituents silychristin and hyperoside in column eluent obtained from *Hemidesmus indicus* and *Ichnocarpus frutescens*, respectively. The chromatograms are displayed in Figures 3.10 and 3.11, each illustrating a comparison between pure standards and the respective plant column eluent.

Figure 3.10.A shown the HPLC chromatogram of pure silychristin, with a distinct peak at a retention time of 5.5 minutes. This peak represents the characteristic elution time of pure silychristin under the specific chromatographic conditions used in this analysis. Figure 3.10.B displays the HPLC chromatogram of the column eluent from *Hemidesmus indicus.* A similar peak is observed at approximately 5.5 minutes, which aligns closely with the retention time of the pure silychristin. The presence of this peak at the same retention time strongly suggests that silychristin is present in the *Hemidesmus indicus*. The matching retention times between the pure silychristin and the column eluent from *Hemidesmus indicus* confirm the successful identification of silychristin.

Figure 3.10B also shows several additional peaks at earlier retention times (approximately between 1 and 5 minutes) that are absent in the chromatogram of pure silychristin (Figure 3.10A). These additional early retention peaks are primarily flavonoids and phenolic compounds, consistent with the flavonoid-rich fraction of *H. indicus* used for silica gel chromatography. Their multiple hydroxyl and methoxy groups increase polarity, causing weak interaction with the non-polar reversed-phase HPLC column and resulting in earlier elution compared to less polar compounds (Harborne and Williams, 2000; Singh et al.,

2017). Literature reports also identify simple phenolics and aromatic aldehydes in *H. indicus* root, including vanillin, hydroquinone, and 2-hydroxy-4-methoxybenzaldehyde (MBALD) (Ravishankara et al., 2002; Swathi et al., 2019b). These low molecular weight, highly polar molecules are expected to elute early under reversed-phase HPLC conditions and likely account for some of the additional early chromatographic peaks observed.

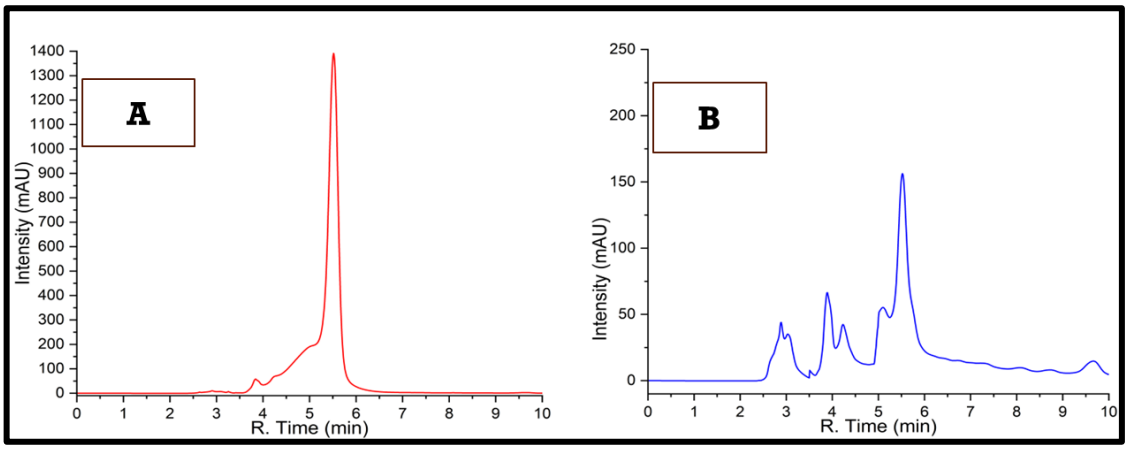

Figure 3.10: HPLC Chromatogram of (A) Pure silychristin, showing a single peak at around 5.5 minutes; (B) Column eluate from *Hemidesmus indicus*, displaying a similar peak at around 5.5 minutes, representing silychristin present in the *Hemidesmus indicus*.

Figure 3.11.A shows the HPLC chromatogram of pure hyperoside, with a significant peak appearing at a retention time of 2.88 minutes. This peak is indicative of the specific elution time of pure hyperoside under the conditions used. Figure 3.11.B presents the HPLC chromatogram for the column eluent from *Ichnocarpus frutescens*. In this chromatogram, a peak is observed at around 2.88 minutes, matching the retention time of the pure hyperoside standard. The consistent retention time between the pure hyperoside and the column eluent from *Ichnocarpus frutescens* suggests that the compound isolated from *Ichnocarpus frutescens* is indeed hyperoside. Thus, the HPLC analysis effectively confirmed the presence of silychristin in *Hemidesmus indicus* and hyperoside in *Ichnocarpus frutescens*. The matching retention times between the column eluent and their corresponding pure standards validate the identity of these phytoconstituents.

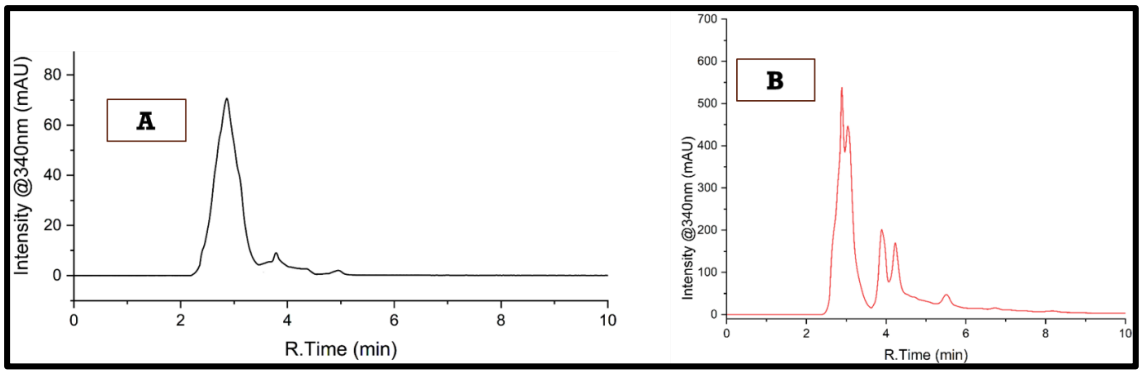

Figure 3.11: HPLC Chromatogram of (A) Pure hyperoside, showing a single peak at around 2.88 minutes; (B) Column eluate from *Ichnocarpus frutescens*, displaying a similar peak at around 2.88 minutes, representing hyperoside present in the *Ichnocarpus frutescens*.

3.3.7.3 NMR spectroscopy

NMR spectroscopy was utilized to confirm the structural identities of hyperoside and silychristin isolated from column eluents. ^{1}H NMR spectrum of hyperoside (Figure 3.12) displayed multiple peaks corresponding to different proton environments within the molecule. Aromatic protons were observed in the 6.0-8.0 ppm region, indicating the flavonoid structure. A prominent peak around 5.0 ppm suggests the presence of an anomeric proton, characteristic of the sugar moiety, while peaks in the 3.0-4.0 ppm range further confirm the glycosidic linkage. These features align with the known structure of hyperoside. Similarly, the ^{1}H NMR spectrum of silychristin (Figure 3.13) reveals aromatic protons in the 6.0-8.0 ppm region, indicative of its flavonoid structure. The spectrum also shows a peak around 5.0 ppm and signals in the 3.0-4.0 ppm range, consistent with the presence of a methoxy group and a sugar moiety, validating the identity of silychristin.

The ^{13}C NMR spectrum of hyperoside (Figure 3.14) features peaks between 110-160 ppm, corresponding to aromatic carbons, and signals around 60-100 ppm, indicative of sugar carbons. These chemical shifts confirm the flavonoid and sugar components of hyperoside. The ^{13}C NMR spectrum of silychristin (Figure 3.15) similarly shows aromatic

carbon peaks in the 110-160 ppm range and additional signals in the 60-100 ppm range, consistent with its structural features. Thus, both ^{1}H NMR and ^{13}C NMR spectra provide conclusive evidence of the presence of hyperoside and silychristin in the isolated fractions of *Ichnocarpus frutescens* and *Hemidesmus indicus* respectively.

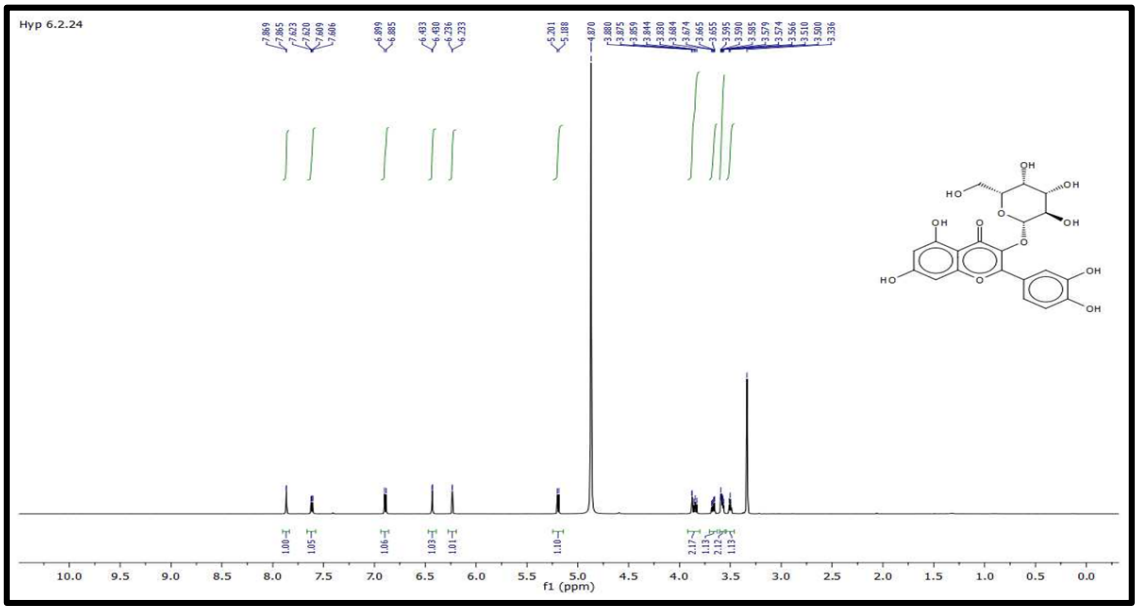

Figure 3.12: ^{1}H NMR spectrum of hyperoside (column eluents from *Ichnocarpus frutescens*)

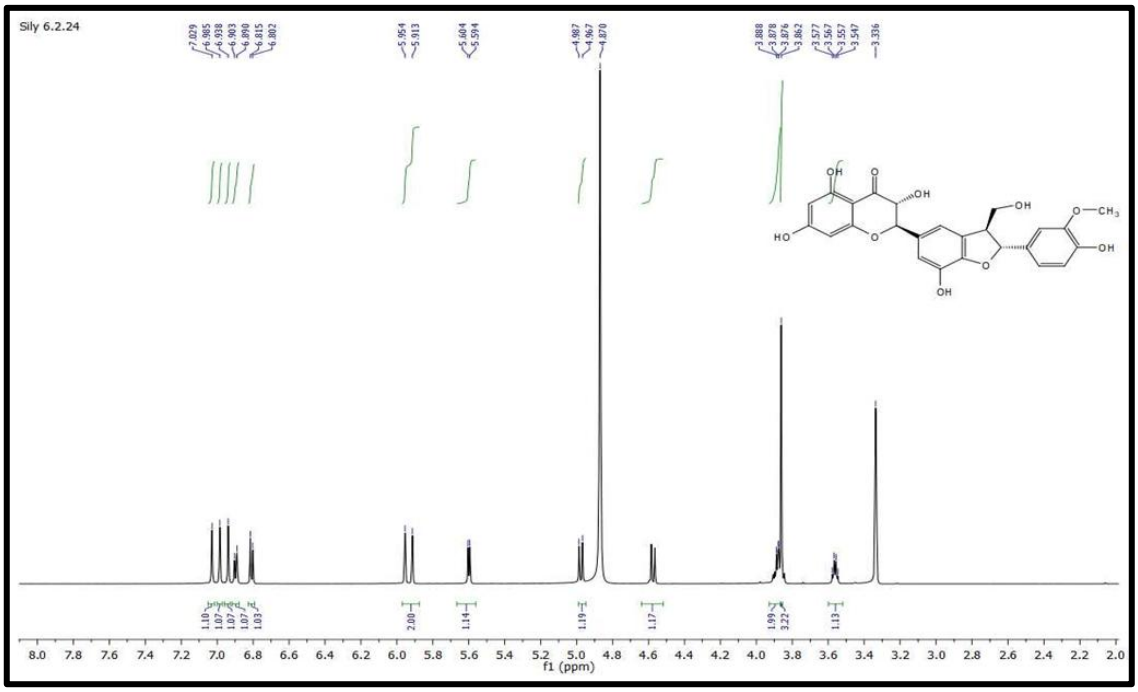

Figure 3.13: ^{1}H NMR spectrum of Silychristin (column eluents from *Hemidesmus indicus*)

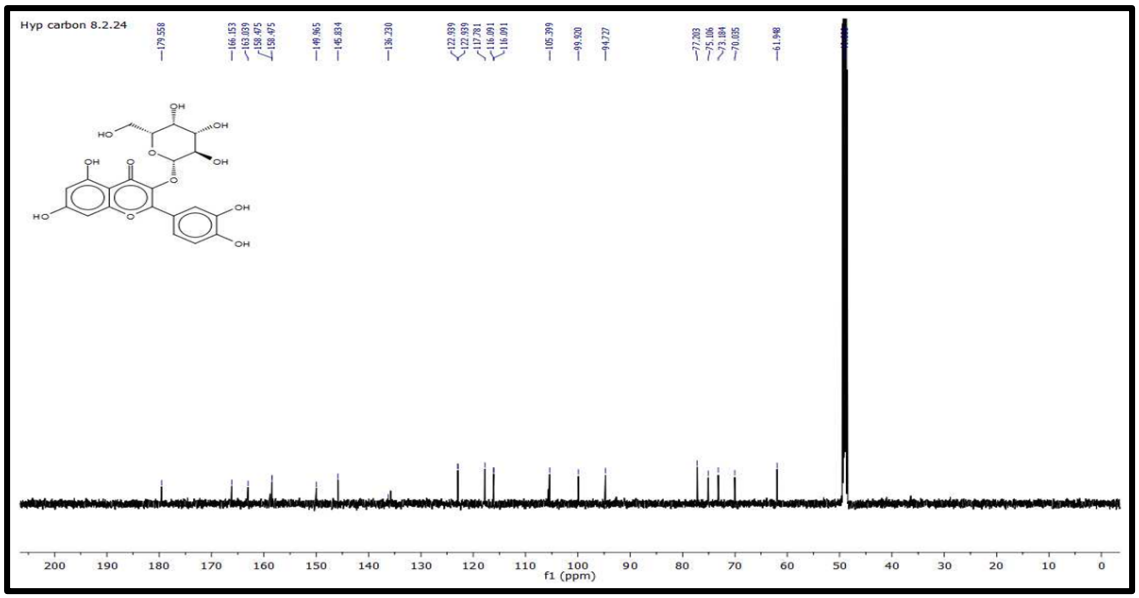

Figure 3.14: ^{13}C NMR spectrum of hyperoside (column eluents from *Ichnocarpus frutescens*)

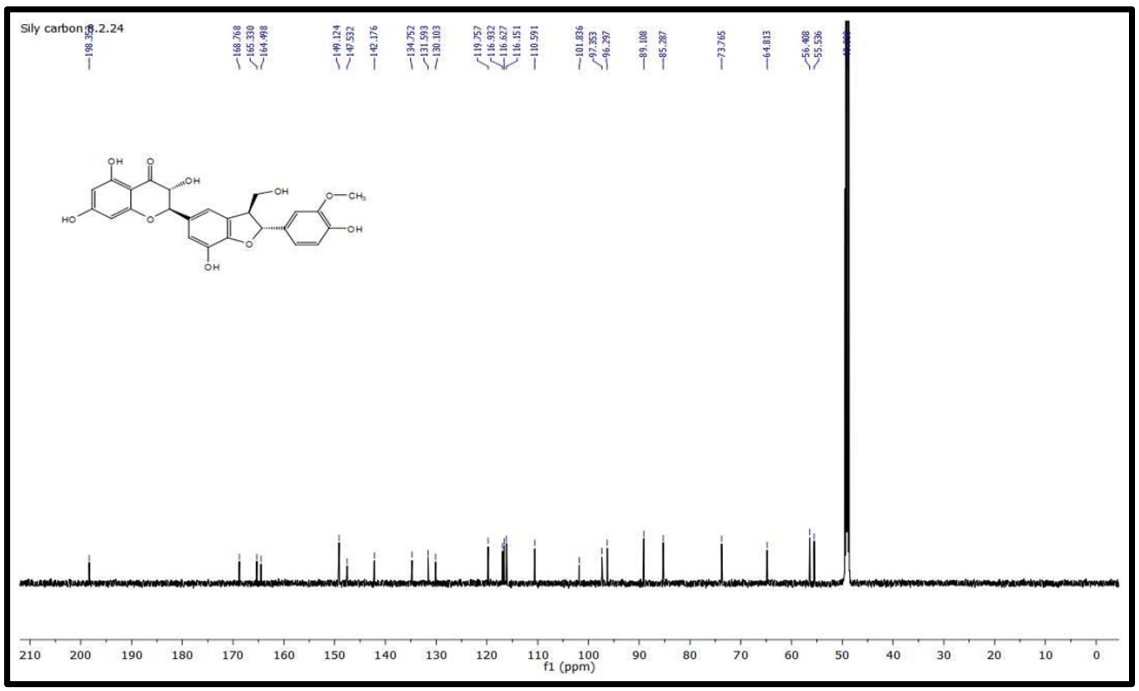

Figure 3.15: ^{13}C NMR spectrum of Silychristin (column eluents from *Hemidesmus indicus*)

3.3.7.4 High-Resolution Mass Spectrometry (HR-MS).

HR-MS was used to confirm the molecular weights and structural identities of the compounds silychristin and hyperoside. The accurate mass measurements provided by HRMS enable precise determination of the molecular formulas of these

phytoconstituents. In Figure 3.16, the HRMS spectrum of silychristin shows a prominent peak at an m/z value of 481.1168, which corresponds to the $[M+H]^-$ ion. This m/z value is consistent with the molecular formula $C_{25}H_{22}O_{10}$, aligning with the expected molecular weight of silychristin. The high accuracy of this measured value confirms the identity of the compound as silychristin, supporting the structural information obtained from ^{1}H and ^{13}C NMR spectroscopy. Figure 3.17 represented the HRMS spectrum for hyperoside, where a significant peak was observed at an m/z value of 463.0972, corresponding to the $[M+H]^-$ ion.

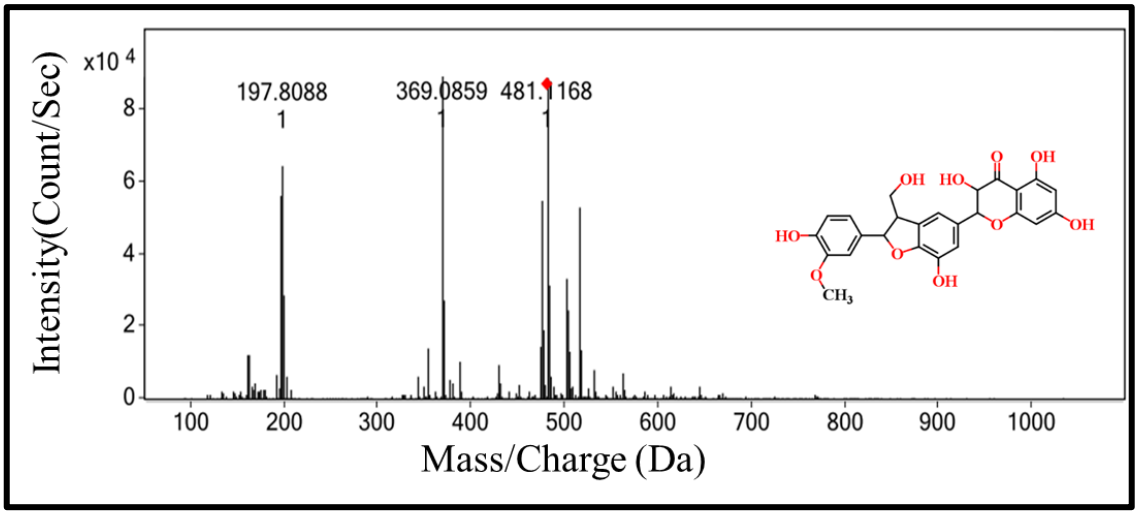

Figure 3.16: HRMS spectrum of silychristin (column eluents from *Hemidesmus indicus*) showing a prominent peak at an m/z value of 481.1168.

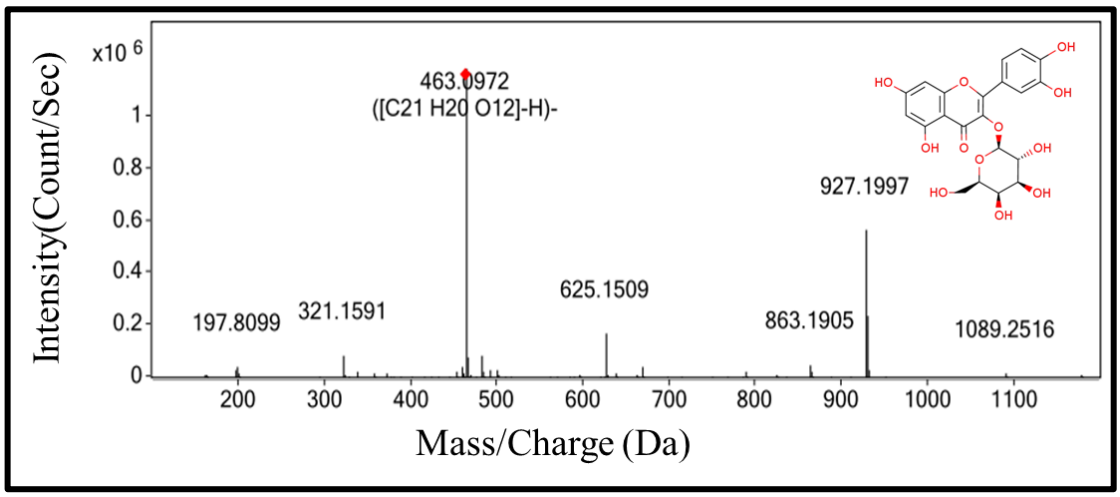

Figure 3.17: HRMS spectrum for hyperoside (column eluents from *Ichnocarpus frutescens*), showing a prominent peak at an m/z value of 463.0972.

This peak aligns with the molecular formula $C_{21}H_{20}O_{12}$, which is indicative of hyperoside. The accurate mass measurement thus confirms the identity of hyperoside, consistent with the structural data from NMR analysis. This mass spectrometry data, combined with NMR results, validated the successful isolation and identification of silychristin and hyperoside compounds from *Hemidesmus indicus* and *Ichnocarpus frutescens,* respectively.

3.4 Conclusion

This chapter focused on the successful isolation and identification of key phytochemicals from the roots of *Hemidesmus indicus* and *Ichnocarpus frutescens*. Both plants were found to contain several classes of phytochemicals, including tannins, flavonoids, phenolics, terpenoids, steroids, and alkaloids, with methanol being the most effective solvent for extracting these compounds. Methanol yielded the highest extractive yield, total phenolic content, and total flavonoid content, followed by water. The highest total phenolic contents were 154.15 mg GAE/g for *Hemidesmus indicus* and 137.00 mg GAE/g for *Ichnocarpus frutescens*, while the highest total flavonoid contents were 70.61 mg QE/g and 52 mg QE/g, respectively. Elemental analysis revealed that *Hemidesmus indicus* had a higher nitrogen content, often associated with amino acids, proteins, and alkaloids, whereas *Ichnocarpus frutescens* had a higher carbon content. Both extracts contained similar hydrogen percentages. Inductively Coupled Plasma Atomic Emission Spectroscopy detected several elements, including Al, Ca, Fe, K, Mg, Na, Cu, Li, Mn, S, Zn, P, and Si, in both plants. To isolate specific phytoconstituents from *Hemidesmus indicus* and *Ichnocarpus frutescens*, silica gel column chromatography was utilized. The flavonoid yield from the roots of *Hemidesmus indicus* and *Ichnocarpus frutescens* was 13.54% w/w and 9.23% w/w, respectively, using the Aqueous Two-Phase System (ATPS). Through silica gel column chromatography, silychristin was isolated from *Hemidesmus*

indicus (fractions 4-9) and hyperoside from *Ichnocarpus frutescens* (fractions 13-17), with yields of 0.21% w/w and 0.37% w/w, respectively. Silychristin was identified as a whitish solid with a distinctive odour, whereas hyperoside was characterized as a yellow solid with a characteristic odour. Thin Layer Chromatography confirmed the presence of these compounds, with retention factors (Rf) of 0.62 for hyperoside and 0.97 for silychristin, consistent with their respective standards. High-Performance Liquid Chromatography further validated these findings, showing retention times of 5.5 minutes for silychristin and 2.88 minutes for hyperoside, matching those of the pure compounds. NMR spectroscopy provided additional structural confirmation. The ^{1}H NMR spectra revealed characteristic peaks for aromatic protons and sugar moieties, while the ^{13}C NMR spectra indicated the presence of aromatic and sugar carbons, consistent with the structures of silychristin and hyperoside. High-Resolution Mass Spectrometry offered precise molecular weight data, showing peaks at m/zm/z 481.1168 for silychristin and 463.0972 for hyperoside. These values correspond to the molecular formulas $C_{25}H_{22}O_{10}$ for silychristin and $C_{21}H_{20}O_{12}$ for hyperoside, confirming their identities.

Therefore, the combined use of TLC, HPLC, NMR, and HRMS successfully identified and confirmed the presence of silychristin and hyperoside in *Hemidesmus indicus* and *Ichnocarpus frutescens.* These findings provide valuable insights into the phytochemical composition of these plants and set the stage for exploring their antidiabetic, antioxidant, and anticancer potential in the next chapter, integrating traditional knowledge with modern scientific techniques.