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# EXPLORING THE PHYTOCHEMICAL PROPERTIES AND ANTICANCER ACTIVITY ETHANOLIC EXTRACT OF C. INFUNDIBULIFORMIS

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## ABSTRACT

Herbal medicine, also known as phytotherapy or botanical medicine, is an ancient healing practice that uses the therapeutic properties of plants and plant-based substances to promote health and treat various ailments. Phytochemical properties and anticancer activity of *C. infundibuliformis* (L.) ethanolic extract collected from Jamia Salafiya Pharmacy College Campus in Kerala, South India. *C. infundibuliformis* (L.) Authentication by experts from the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, and deposition in the Fischer Herbarium (accession number 25233) ensured the reliability of the collected samples. Physical, chemical, qualitative, quantitative, and MTT assays of the ethanolic extract were performed using the standard protocol. The ethanolic extract of *C. infundibuliformis* had a semi-solid consistency, distinctive brown colour, and a yield percentage of 8.67, suggesting the presence of valuable bioactive compounds. Qualitative analysis revealed the presence of steroids, triterpenoids, flavonoids, phenolic compounds, and tannins, indicating their rich chemical composition with potential pharmacological significance. Quantitative assessments estimate the phenolic compound and flavonoid concentrations and provide valuable insights. The total terpenoid content, determined using the lupeol technique, highlighted the presence of terpenoids in the extract. LC-MS analysis identified various compounds that contribute to the potential therapeutic properties of the plant. The study extended the cytotoxic effects on SK-MEL-3 skin cancer cells, with the MTT assay demonstrating an inhibitory effect and $IC_{50}$ values falling within the range of 62.5 to 1000 µg/ml. The AO/EB staining technique visually represented morphological alterations in treated cells, indicating the potential apoptotic effects induced by the extract. This comprehensive analysis of *C. infundibuliformis* (L.) not only elucidates its chemical composition but also underscores its potential therapeutic applications, particularly in cancer treatment. This study sets the stage for further exploration and development of pharmaceuticals derived from this natural source.

**KEYWORDS:** Cancer, Phytotherapy, *C. infundibuliformis*, Cytotoxicity, AO/EB.

## INTRODUCTION

Nature has always served as a testament to this remarkable phenomenon of symbiosis. Historically, it has provided an abundance of medicinal plants, underscoring the evolution of traditional medicine.[1,2] This practice has been a continuous pursuit of humanity to discover innovative remedies for ailments, a tradition that melds seamlessly with modern medicine. India has a rich heritage in terms of traditional medicine. Its medical traditions encompass various systems such as Ayurveda, Siddha, and Unani. Recently, there has been renewed interest in alternative Indian medicinal systems and the therapeutic potential of natural products, particularly those sourced from plants. From time immemorial to today, plants have been fundamental in medicine.[3] The World Health Organization (WHO), headquartered in Geneva, notes that approximately 80% of the population in developed countries depends on traditional or alternative medicine for primary health care. A significant portion of this traditional medicine relies heavily on plant extracts and their active compounds.

Herbal medicine, also known as phytotherapy or botanical medicine, is an ancient healing practice that uses the therapeutic properties of plants and plant-based substances to promote health and treat various ailments.[4,5] This traditional form of medicine has been an integral part of human culture for millennia, dating back to the early civilisations. Herbal medicine draws

upon the wisdom of indigenous cultures, historical texts, and modern scientific research to harness the healing potential of nature's bounties. Herbal remedies encompass a vast array of plants, herbs, roots, flowers, and fungi, each containing unique chemical compounds that contribute to their medicinal properties. These natural compounds can be used to address a wide range of health concerns, from common ailments, such as colds and digestive issues, to chronic conditions, such as antibacterial and wound healing. Herbal medicine spans the globe, with different cultures developing unique herbal traditions. Traditional Chinese Medicine, Ayurveda from India, Native American herbalism, and European herbalism are just a few examples of diverse herbal systems that have evolved over centuries.[6] Phytochemical compounds, derived from plants, have garnered considerable attention for their potential anticancer activities.

These naturally occurring substances encompass a diverse array of chemical compounds, each exhibiting unique properties that contribute to their therapeutic potential against cancer. The exploration of phytochemicals as anticancer agents stems from the rich chemical diversity found in plant sources, offering a vast repertoire of compounds with varying mechanisms of action.

## MATERIAL AND METHODS

### The acquisition and authentication of *C. infundibuliformis* (L.)

*C. infundibuliformis* (L.) plants were collected at the Jamia Salafiya Pharmacy College Campus, Pulikkal, Malappuram District, Kerala, South India, and subjected to thorough authentication by a plant authentication expert from the Institute of Forest Genetics and Tree Breeding, Coimbatore. A sample specimen was deposited in the Fischer Herbarium under the accession number 25233. Freshly harvested leaves were air-dried in the shade. The dried leaves were finely ground using a mechanical grinding machine, and the resulting powder was sifted through a mesh of size 60 to achieve the desired particle size. The reduced-size plant material was carefully stored in an airtight container for the duration of this study.[7]

### The pharmacognostic examination of *C. infundibuliformis* (L.).

Pharmacognostic studies of plant products involve a comprehensive understanding of various aspects, including the plant's habitat, the characteristics of its powdered or incomplete parts, the specific plant organ used, and the chemical properties of the plant components responsible for the therapeutic effects. To ensure substance identity and purity, initial steps, such as macroscopic and microscopic examinations of the plant, are crucial and should be conducted before any other experiments. Without the correct verification of the authenticity of the plant product, its therapeutic efficacy is compromised. Recently, there has been a growing demand for the pharmacognostic standardisation of medicinal plants. This study aimed to standardise *C. infundibuliformis* (L.) pharmacognostically, which will aid in the accurate identification and verification of its medicinal properties.

### Phytochemical evaluation

Phytochemistry is a branch of science dedicated to understanding the diverse range of secondary metabolites that are synthesised by plants. These bioactive and intricately structured molecules are valuable resources in the plant kingdom. The presence of a wide array of phytochemicals in plants renders herbal medicines unique. The medicinal and physiological benefits of these herbal remedies stem from the synergistic effects of their phytoconstituents. While many plant chemicals exist, only a limited portion have been quantified, and even fewer have undergone pharmacological or biological screening. In the case of *C. infundibuliformis* (L.), this study aimed to comprehensively analyse and identify the various phytoconstituents present in the entire plant, separate these phytoconstituents, and characterise them through structural analysis.[8]

### Preparation of the ethanolic extraction

*C. infundibuliformis* (L.) Ethanolic Soxhlet extraction is a specific method employed to extract bioactive compounds from *C. infundibuliformis* (L.) plant material using ethanol (ethyl alcohol) as the solvent and the Soxhlet extraction technique. This process allows for efficient extraction of various phytochemicals, secondary metabolites, and potentially medicinal compounds from plants. The plant material, often leaves or other relevant parts, is first finely ground or powdered to increase the surface area and facilitate extraction. Finely ground plant material is loaded into a porous thimble, which is typically made of cellulose or glass microfibres. The Soxhlet extraction apparatus consisted of a Soxhlet extractor (containing the sample thimble), a flask to hold the ethanol solvent, and a condenser. The thimble containing the sample was positioned above the flask. In this process, ethanol is heated in the flask, causing it to vaporise and rise through a vertical reflux system. As it condenses in the condenser, ethanol drips back into the thimble containing the plant material. This cycle continued, allowing the solvent to continuously extract various compounds from the plant material. The condensed extract, which contains dissolved phytochemicals and bioactive compounds from *C. infundibuliformis* (L.), accumulates in flasks. The extraction process was repeated until the desired level of extraction was achieved, which may vary depending on the specific target compound. Ethanolic Soxhlet extraction is chosen when ethanol is suitable for extracting a range of compounds from *C. infundibuliformis* (L.) for various purposes, such as pharmaceutical or herbal applications. Ethanol is known for its ability to dissolve both polar and non-polar compounds, making it versatile for the extraction of a wide spectrum of phytoconstituents. The solvent was

selected based on the specific compounds of interest and their solubility. Additionally, safety precautions, including proper ventilation and adherence to flammability guidelines, were followed when working with ethanol owing to its flammable nature.[8]

**Qualitative analysis - chemical tests**

The plant extracts which were obtained using an organic ethanol solvent, and water extracts of *C. infundibuliformis* (L.) obtained via the continuous hot process using a Soxhlet apparatus were checked as follows:

**Test for alkaloids**

A small portion of the plant extract was mixed with a limited amount of hydrochloric acid (HCl) and filtered. The resulting filtrate underwent a series of alkaloidal tests using various alkaloidal solution reagents, including Mayer's reagent, Dragendorff's reagent, Hager's reagent, and Wagner's reagent, for further analysis.

**Test for the identification of carbohydrates**

A small portion of the extract to be tested was dissolved using 5 ml of water and then filtered. The filtrate was then tested for the presence of carbohydrates using the following tests:

**Molisch's test**

Two or three drops of one percent alpha alpha-naphthol solution were added to a small portion of the filtrate. About 2 ml of Conc. $H_2SO_4$ was gently added along the sides of the test tubes. A ring which is violet in colour formed between the intersection of the two layers suggests the existence of carbohydrates in the sample.

**Fehling's test**

The filtrate was treated with a small amount (1 ml) of Fehling 's solutions A and B, and the substance was heated for approximately 5-10 minutes in a boiling water bath. The presence of a dark red residue at the bottom suggests the presence of carbohydrates.

**Detection of glycosides**

**Test for Cardiac glycoside**

**Keller-Killani test**

Glacial acetic acid containing one drop of 5 per cent ferric chloride was applied to a small portion (2 ml) of the extract. In the solution, conc. sulphuric acid was then added to the mixture. The reddish-brown colour appeared at the junction of the two liquids and later turned bluish-green, suggesting the presence of cardiac glycosides in the sample.

**Test for identification of anthraquinone glycosides**

**Borntrager's test**

Approximately 3 ml of the extract was added to the dil. $H_2SO_4$. The resulting solution was then heated, filtered, and cooled. Equal amounts of chloroform were then added to the filtrate. The chloroform layer was removed from the mixture via separation. Ammonia solution was then added to this solution. Ammonia layer will acquire pink colour.

**Test for identification of saponin glycosides**

**Foam test**

The presence of foam was tested by placing different crude drug powders in a test tube with water. The mixture was shaken aggressively. The formation of foam and its amount indicates the presence of saponins.

**Test for identification of coumarin glycosides**

Alkali solution was added to the small part of our extract to make it alkaline. The presence of coumarin glycosides was determined based on the colour of the fluorescence. The fluorescence colour formed may be blue or green.

**Identification of phytosterol**

Approximately 1 g of extract was dissolved in a few drops of acetic acid. The extract was blended with 3 ml of acetic anhydride, followed by the addition of a few drops of concentrated sulphuric acid. The presence of phytosterols suggests the appearance of a bluish-green colour.

**Detection of fats and oils**

A limited volume of each extract was placed individually between two sheets of normal filter paper. Oil staining was performed on filter paper. This indicates the presence of fixed oil. Add 0.5 N alcoholic KOH and phenolphthalein were added to a small number of extracts. The entire material was heated for 1-2 hours on a water table. Soap production showed the presence of both fixed oils and fats in our sample.

**Test for the identification of tannins and phenolic substances**

Separately a limited number of different extracts were dissolved in $H_2O$ and tested for existence of phenolic compounds and tannins (Eghdami A, Sadegha F 2010). The development of violet colour with the application of a 5 percent dilute solution of ferric chloride suggests the presence of phenolic compounds in the sample under study. The development of a white precipitate with the application of 1 per cent gelatin solution and 10 per cent sodium chloride showed the presence of phenolic substances in the sample under study. The formation of a white precipitate with the addition of 10% lead acetate solution revealed the existence of phenolic substances in the sample analysed in this study.

**Test for proteins**

The extracts were dissolved in a small amount of water, and the following tests were performed to determine the presence of proteins in the sample.

**Millon's test:** The presence of red colour indicates the presence of proteins and free amino acids in the sample.

**Biuret test:** Equal amounts of 5% sodium hydroxide solution and 1% copper sulphate solution were added.

The presence of proteins and free amino acids is indicated by the appearance of a pink or purple colour.

**Test for identification of mucilages and gums**

Approximately 10 ml of each extract was applied separately with continuous stirring to 25 ml of pure alcohol and purified. The precipitate was air-dried and tested for the presence of carbohydrates and their swelling effects.

**Test for identification of flavonoids**

(i) The Addition of NaOH solution will lead to the development of blue to violet colour which specifies the existence of Anthrocyanins; yellow colour indicates the presence of flavones, and yellow to orange indicates the presence of flavanones.

(ii) Addition of Conc. $H_2So_4$, leads to the formation of yellowish-orange colour in the sample containing Anthocyanins and orange to crimson colour in the sample containing Flavonones.

(iii) Shinoda's test – Different extracts were dissolved in alcohol, to which firstly a piece of magnesium ribbon was added and then Conc. HCl was added dropwise and then heated. The existence of flavonoids is indicated by the emergence of magenta colour in the solution.

**Test for lignin**

Add the alcoholic solution of phloroglucinol and the strong hydrochloric acid to a limited volume of the sample. Then heat the mixture. The production of lignin is shown by the existence of red colour.

**Identification of terpenoids**

To the small portion of the plant extract which is under study, add tin and thionyl chloride. The resultant mixture was gently and carefully warmed. The development of pink colour suggests the presence of triterpenoid compounds.

**Test for steroids**

A small portion of the extract was taken for the analysis. To this (2 ml of the extract) chloroform was added and mixed well. To this solution about 1 to 2 ml of acetic anhydride was added. About 2 drops of concentrated sulphuric acid was added to through the sides of the test tube in the solution mixture. The presence of steroids in the sample will be indicated by the initially red colour which will then turn to blue and finally it becomes green colour appears. This colour change indicates the presence of steroids in the sample.

**Phytochemical quantitative estimates**

**Estimation of Phenols**

In the estimation of phenolic compounds using the Folin-Ciocalteu assay, a series of standard solutions ranging from 20 to 100 µg/ml of gallic acid were prepared to establish a calibration curve. Sample extracts, obtained through meticulous solvent extraction, were treated with the Folin-Ciocalteu reagent and sodium carbonate solution. After a 2-hour incubation period, the resulting solutions exhibited a distinct blue color. The absorbance of these solutions, as well as the standard solutions, was measured at 750 nm using a spectrophotometer. The absorbance values obtained from the standard solutions were then used to construct a calibration curve. By comparing the absorbance of the sample extract to this curve, the total phenolic content was quantified. For instance, the phenolic content in the sample was determined to be 45 mg of gallic acid equivalents per gram of extract. This method, with its rigorous calibration and measurement steps, offers a precise and quantitative assessment of phenolic compounds in the analyzed sample, providing valuable information about its potential health-promoting properties.

**Estimation of Terpenoids**

To analyze the extract, 1 mg was dissolved in 1 ml of methanol and combined with 150 µl of 5% Vanillin-glacial acetic acid and 500 µl of perchloric acid solution. The mixture underwent heating at 60°C for 45 minutes, followed by cooling in an ice water bath to reach ambient temperature. After adding 2.25 ml of glacial acetic acid, the absorbance of each sample solution was measured at 548 nm using a UV-visible spectrophotometer. Linalool standards (100, 200, 400, 800, 1000 µg/ml) in methanol served as references, and the results were expressed as mg Linalool equivalents. This analytical procedure allows for the quantification of Linalool content in the sample extract.

**Estimation of Flavonoids**

For the assessment of total flavonoid content, the aluminum chloride colorimetric assay was employed. In a 10 ml volumetric flask, a mixture of 1 mg of extract and 4 ml of distilled water was prepared. To this flask, 0.30 ml of 5% sodium nitrite was added, followed by the addition of 0.3 ml of 10% aluminium chloride after 5 minutes. Subsequently, 2 ml of 1M sodium hydroxide was introduced, and the volume was adjusted to 10 ml with distilled water. Reference standard solutions of Quercetin (20, 40, 60, 80, and 100 µg/ml) were also prepared using the same procedure. The absorbance for both test and standard solutions was determined against the reagent blank at 510 nm using a UV/Visible spectrophotometer. The total flavonoid content was expressed as µg of quercetin equivalents per milligram of extract. This method allows for the quantification of flavonoids in the sample using a standardized reference.

**Phytochemical evaluation of ethanolic extract of *C. infundibuliformis* (L.) by LC-MS**

*C. infundibuliformis* (L.) leaf ethanolic extract phytochemical analysis was studied by using the LC-MS. The chemical constituents of the ethanolic extracts were determined using LC-MS. LC-MS analysis was performed using Mariner Bio spectrometry equipped with a binary pump. The HPLC was interfaced with a Q-TOF mass spectrometer fitted with an ESI source. Full-scan mode from m/z 100 to 1200 was performed with a

source temperature of 140°C. HPLC column Phenomenex 5µ C8, (150 × 2 mm i.d.) was used for the analysis. Solvent was methanol with 0.3% formic acid. Solvents were delivered at a total flow rate of 0.1 mL/min. The solvent was run by isocratic elution. The MS spectra were acquired in the positive ion mode.[9]

***In-vitro* anticancer activity of *C. infundibuliformis* ethanolic extract**

To enumerate active raw cells, a hemocytometer was employed in this study. These active cells were subsequently seeded into sterile 96-well plates at a concentration of 1 × 10^4 cells/ml in each well and allowed to incubate for a duration of 24 hours to facilitate their adherence and growth. Subsequently, the breast cancer cells were subjected to treatment with varying concentrations of *C. infundibuliformis* ethanolic extract, spanning from 50 to 2.5 µl/ml. Following this treatment, the plates were incubated at a temperature of 37°C for 24 hours under conditions of 95% humidity in sterile air, supplemented with 5% CO2. Subsequent to this incubation period, the cells were subjected to treatment with the MTT reagent.[10] The percentage of cell viability was determined using the subsequent formula:

Half-maximal inhibitory concentration ($IC_{50}$)

$$\frac{A_c \text{ control} - A_s \text{ of treated X } 100}{A_c \text{ control}}$$

***C. infundibuliformis* ethanolic extract apoptotic induction was measured by acridine orange/ethidium bromide (AO/EB) staining**

Raw cells were initially seeded in sterile six-well plates at a concentration of $5 \times 10^4$ cells per well and then incubated for a period of 24 hrs. Following this incubation, the cells underwent a thorough washing with PBS for 24 hrs, effectively eliminating any deceased or undetected cells from the culture. Subsequently, these cells were subjected to treatment with varying concentrations of *C. infundibuliformis* ethanolic extract, ranging from 125 µg/ml down to 7.785 µg/ml. After an additional 24-hour incubation period, the cells were gently detached and subjected to further washing with sterile PBS. Any cells that did not detach were then exposed to a staining solution composed of AO/EB (100 µg/ml) in a 1:1 ratio for a duration of 5 minutes at room temperature. Subsequently, these AO/EB-stained cells were meticulously examined under a fluorescence microscope at a 45x magnification to facilitate the identification of live and dead cells. The number of cells exhibiting characteristics indicative of apoptosis was quantified in relation to the total cell population observed within the microscope's field of view. This experimental approach allowed for the assessment of the impact of varying concentrations of *C. infundibuliformis* ethanolic extract on raw cells, with particular attention paid to the induction of apoptosis-related changes in the cell population.[11]

## RESULTS AND DISCUSSION

**Plant collection and Authentication**

*C. infundibuliformis* (L.) plants were collected at the Jamia Salafiya Pharmacy College Campus, Pulikkal, Malappuram District, Kerala, South India, and subjected to thorough authentication by a plant authentication expert from the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore. A sample specimen was deposited in the Fischer Herbarium under the accession number 25233 (Figure 1).

**Figure 1: *C. infundibuliformis* (L.).**

***C. infundibuliformis* (L.) selected medicinal plant collection and extraction**

In the investigation of the extractive values of ethanolic solvents from *C. infundibuliformis* (L.), various physical and chemical characteristics were meticulously recorded to unveil the intricate properties of the extracted substance. The sample, identified by the code C. infundibuliformis (L.), underwent extraction using ethanol as the solvent. The resulting consistency of the extract was noted as semi-solid, indicative of a substance possessing qualities between liquid and solid states. The extract exhibited a distinctive brown color, suggesting the presence of specific compounds or pigments within the extractive material. The extraction process yielded a percentage of 8.67, reflecting the efficiency of the extraction method in retrieving the desired components from *C. infundibuliformis* (L.). These recorded parameters not only provide a comprehensive understanding of the physical nature of the extract but also contribute valuable insights into the potential bioactive compounds present in *C. infundibuliformis* (L.) that may hold significance in various applications (Table 1).

**Table 1: Extractive values of ethanolic solvents of *C. infundibuliformis* (L.). Physical and chemical characteristics, including colour, consistency, and yield percentages, were recorded.**

| Sample code | Solvent used | Consistency | Extract colour | Yield (%) |
|---|---|---|---|---|
| *C. infundibuliformis* (L.) | Ethanol | Semi- Solid | Brown | 8.67 |

**Qualitative analysis of *C. infundibuliformis* (L.) chemical compounds**

The ethanolic extract of *C. infundibuliformis (L.)* was examined to determine its chemical composition, shedding light on the absence of glycosides, alkaloids, saponins, and fixed oils. Instead, the extract contained steroids, triterpenoids, flavonoids, phenolic compounds, and tannins. Steroids and triterpenoids, which are known for their diverse biological activities, can contribute to the pharmacological potential of the extract (Table 2).

**Table 2: *C. infundibuliformis* (L.) chemical compounds.**

| SI. No | Plant constituent | Ethanolic extract |
|---|---|---|
| 1. | Alkaloids | - |
| 2. | Glycosides | + |
| 3. | Steroids and triterpenoids | + |
| 4. | Flavanoids | + |
| 5. | Phenolic compounds and tannins | + |
| 6. | Proteins | + |
| 7. | Saponins | - |
| 8. | Fixed oils and fats | - |

**Phenols quantitative estimates**

Phenolic compounds, prevalent in all plant tissues, including fruits and vegetables, represent the most common secondary metabolites in plants, characterized by their moderately acidic nature (Table 3).

**Table 3: Absorbance values for different concentrations of standard gallic acid.**

| Concentration(µg/mL) | Absorbance at 750nm |
|---|---|
| 100 | 0.1091 |
| 200 | 0.2411 |
| 400 | 0.5352 |
| 800 | 0.9389 |
| 1000 | 1.0367 |

**Table 4: phenol concentration as gallic acid equivalent g/ mg *C. infundibuliformis* (L.) ethanolic extract.**

| Sample code | Absorbance | Amount of phenol in terms of gallic units (mg) | Amount of phenol in 1mg |
|---|---|---|---|
| CIEE | 0.1612 | 0.104 | 0.52 |

**Flavonoids quantitative estimates**

**Table 5: Absorbance values for different concentrations of standard flavonoid.**

| Concentration (mg/mL) | Absorbance |
|---|---|
| 0.1 | 0.0364 |
| 0.2 | 0.0662 |
| 0.4 | 0.1047 |
| 0.6 | 0.1521 |
| 0.8 | 0.1997 |
| 1 | 0.2257 |

**Table 6: Phenol concentration as quercetin equivalent g/ mg *C. infundibuliformis* (L.) ethanolic extract.**

| Sample code | Absorbance | Amount of flavonoids in terms of quercetin units (mg) | Amount of flavonoids in 1mg sample |
|---|---|---|---|
| CIEE | 0.0744 | 0.252691 | 0.505381 |

**Estimation of total terpenoids content**

The lupeol technique is used to quantify the total flavonoid content in *C. infundibuliformis* (L.) ethanolic extract as quercetin equivalents. The absorbance values for different quantities of standard lupeol and an equivalent quantity of terpenoid extract measured (Table

1). Terpenoid content estimated by equivalents to lupeol.
A stock solution of 1mg/ml lupeol was used.

**Table 7: Absorbance values for different concentrations of standard terpenoid.**

| Concentration(µg/mL) | Absorbance |
|---|---|
| 100 | 0.0452 |
| 200 | 0.068 |
| 400 | 0.1285 |
| 800 | 0.1676 |
| 1000 | 0.1968 |

**Table 8: Terpene concentration in ethanolic and acetone extracts expressed as lupeol equivalent g/ mg of *C. infundibuliformis* (L.) ethanolic extract.**

| Sample code | Absorbance | Amount of terpenoids in terms of lupeol units (µg) | Amount of terpenoids in 1000µg of sample |
|---|---|---|---|
| CIEE | 0.0445 | 22 | 0.270 |

***C. infundibuliformis (L.)* phytochemical analysis by LCMS**

The tentative assignment of compounds detected in the Annona muricata extract via LC-MS analysis is presented. The compounds were detected and identified by their fragmentation patterns in conjunction with PubChem and a research reference article. The peak areas and retention times were used to identify the compounds (Table 1). p-Coumaric acid, quinic acid, apigenin, quercetin-3-O-β-D-glucoside, procyanidin B2, xylopine, β-sitosterol, stephabyssine, butylparaben, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, 1,4-eicosadiene, 1,5-anhydro-d-mannitol, adipic acid, and isobutyl 2-naphthyl ester.

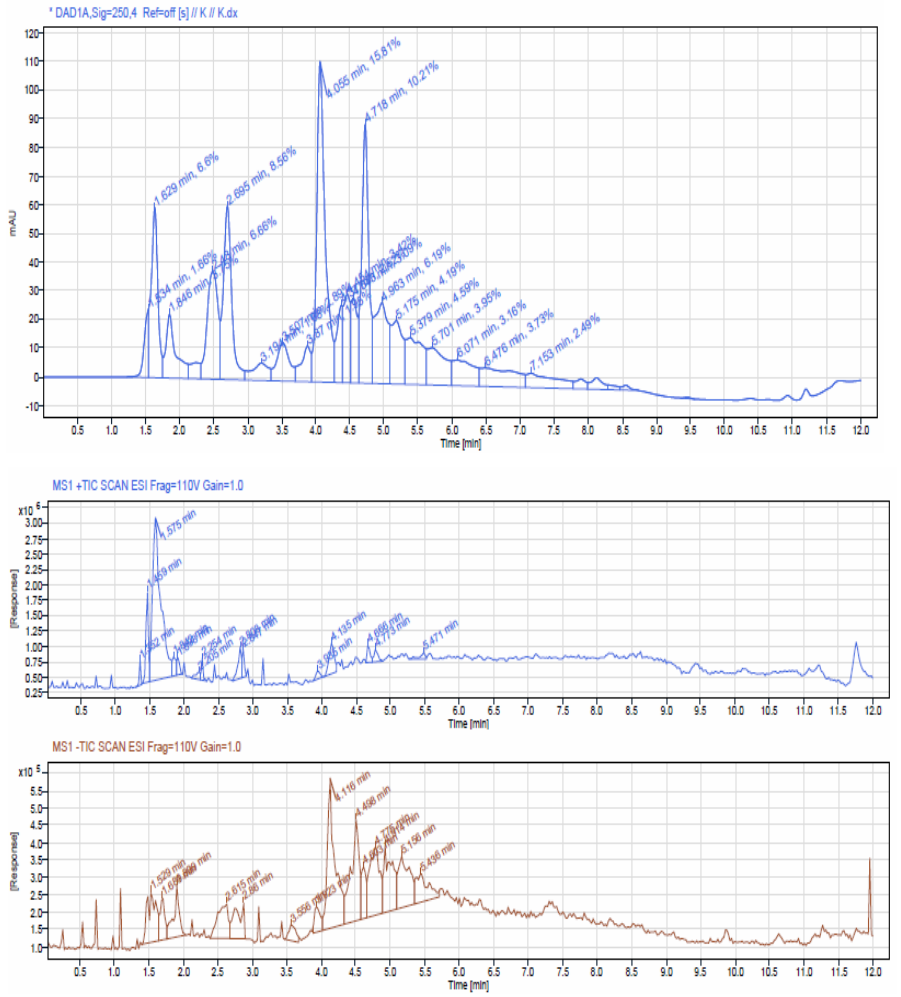


**Figure 2: LC-MS profile of ethanolic extract of *C. infundibuliformis (L.)*.**

**Table 1: Phytochemical analysis of ethanolic extract of *C. infundibuliformis (L.)* By LC-MS analysis.**

| S. NO. | Phytochemicals | m/z |
|---|---|---|
| 1 | p-Coumaric acid | 163 |
| 2 | Quinic acid | 191 |
| 3 | Apigenin | 169 |
| 4 | Quercetin-3-O-β-D-glucoside | 463 |
| 5 | Procyanidin B2 | 577 |
| 6 | Xylopine | 294 |
| 7 | β-Sitosterol | 414 |
| 8 | Stephabyssine | 331 |
| 9 | Butylparaben | 194 |
| 10 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | 330 |
| 11 | 1,4-Eicosadiene | 278 |
| 12 | 1,5-Anhydro-d-mannitol | 164 |
| 13 | Adipic acid, isobutyl 2-naphthyl ester | 328 |

**MTT assay of *C. infundibuliformis* (L.) ethanolic extract**

*C. infundibuliformis* (L.) ethanolic extract were inhibitory effect anticancer skin cancer cell lines SK-MEL-3. It was expressed as (µg/mL). The amount of drug required to stop or kill the 50% of cancer cells called as $IC_{50}$ value. The ability of various substances to inhibit SK-MEL-3 cells was tested by using the *C. infundibuliformis* (L.) ethanolic extract (Figure 2).

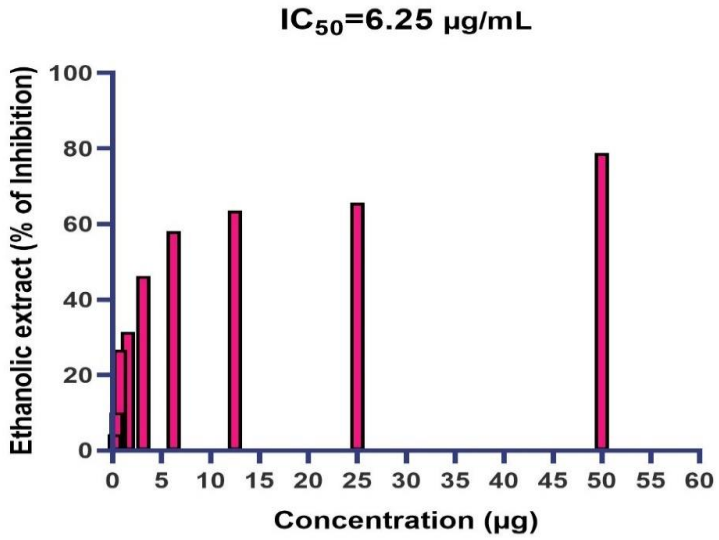


**Figure 2: $IC_{50}$ of skin cancer cells. Data are expressed as the mean value along with the standard deviation, which was computed from three independent experiments. When compared to the control group, *P<0.05, indicating statistical significance.**

The inhibitory potency of the *C. infundibuliformis* (L.) ethanolic extract was evaluated, and the results fell within the range of 62.5 to 1000 µg/ml.

**AO/EB *C. infundibuliformis* (L.) ethanolic extract**

To investigate potential morphological alterations in SK-MEL-3 cells following treatment with *C. infundibuliformis* (L.) ethanolic extract, a fluorescence staining technique utilising acridine orange/ethidium bromide (AO/EB) was employed. This staining method, as depicted in Figure 4, was used to examine and assess the apoptotic characteristics induced by these compounds in SK-MEL-3 cells. AO/EB staining provided valuable insights into cell viability and membrane integrity. This relies on the differential permeability of fluorescent dyes to cells. Live cells typically allow AO to permeate, resulting in green fluorescence, whereas dead cells tend to be more permeable to EB, leading to an orange-red fluorescence. In this study, AO/EB staining fluorescence patterns revealed distinctive cellular states. A) Viable cells characterised by well-organised nuclei emit green fluorescence. B and C early apoptotic cells, showing nuclear condensation, exhibited orange-green fluorescence and late apoptotic cells, with highly condensed or fragmented chromatin, fluoresced in shades ranging from orange to red (Figure 3).

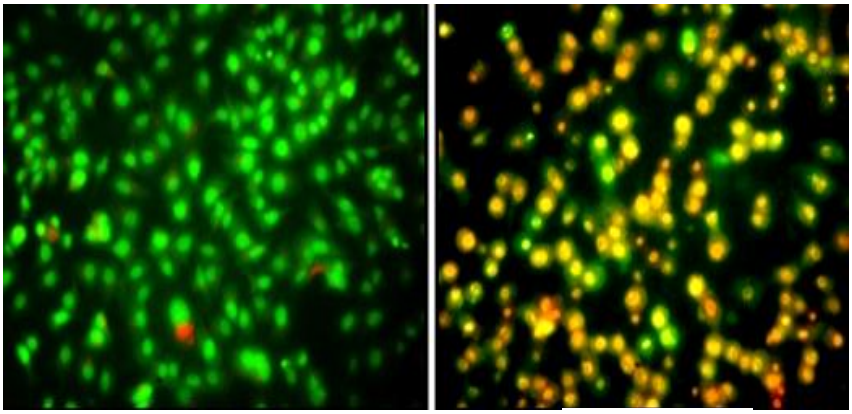

**Figure 3: A: SK-MEL-3 control cells; B: *C. infundibuliformis* (L.) ethanolic extract treated SK-MEL-3 cells.**

The observed variations in cytological and morphological changes within the nuclei, as revealed by AO/EB staining, provide clear indications of the diverse cellular responses triggered by treatment with *C. infundibuliformis* (L.) ethanolic extract. These changes in staining patterns align with well-established and documented apoptotic processes that are frequently observed in cell lines undergoing therapeutic treatments.[12]

Acridine Orange/Ethidium Bromide (AO/EB) staining is a fluorescent dye-based technique widely used in cancer research for the assessment of cell viability and identification of apoptotic and necrotic cells. This staining method provides valuable insights into cellular morphology and viability, making it a crucial tool in cancer studies. The anticancer activities of compounds such as *C. infundibuliformis* (L.) ethanolic extract can be attributed to several mechanisms; however, note that the specific mechanisms on compound its interaction with cancer cells.

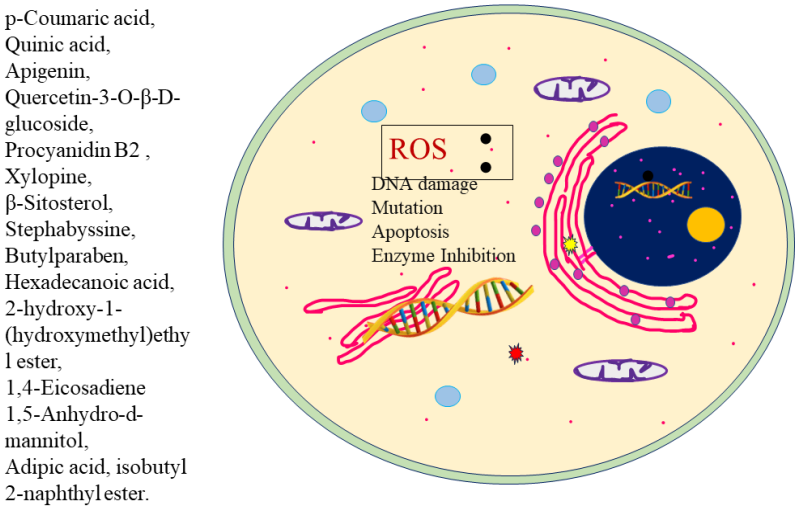


**Figure 4: *C. infundibuliformis* (L.) ethanolic extract anticancer activity possible mechanisms.**

The anticancer activity of phytoconstituents is multifaceted, encompassing diverse mechanisms that impede cancer cell growth and survival. These plant-derived compounds exhibit apoptosis induction, halting the uncontrolled proliferation of cancer cells, and provoke cell cycle arrest. With potent antioxidant properties, they mitigate oxidative stress, a contributing factor to cancer development. Additionally, phytoconstituents showcase anti-angiogenic effects, hindering the formation of blood vessels crucial for tumor sustenance, while their anti-inflammatory attributes reduce the risk of cancer by addressing chronic inflammation. Immunomodulation, interference with DNA repair, inhibition of metastasis, and disruption of key signaling pathways further contribute to their comprehensive anticancer potential. This intricate interplay of mechanisms underscores the promising role of phytoconstituents in the prevention and treatment of cancer (Figure 4).[13]

## CONCLUSION

The study on *C. infundibuliformis* (L.) undertaken at Jamia Salafiya Pharmacy College Campus, Pulikkal, Malappuram District, Kerala, South India, presents a comprehensive analysis of the plant's medicinal potential. The plant underwent rigorous authentication by experts from the Institute of Forest Genetics and Tree

Breeding (IFGTB), Coimbatore, ensuring the reliability of the collected samples. The deposited specimen in the Fischer Herbarium, under the accession number 25233, further establishes a reference for future studies. The investigation into the ethanolic extract of *C. infundibuliformis* (L.) revealed intriguing physical and chemical characteristics, with the semi-solid consistency, distinctive brown color, and a yield percentage of 8.67 indicative of valuable bioactive compounds. Qualitative analysis unveiled the presence of steroids, triterpenoids, flavonoids, phenolic compounds, and tannins, suggesting a rich chemical composition with potential pharmacological significance. Quantitative assessments of phenolic compounds and flavonoids provided valuable insights into their concentrations, with the phenol content estimated as gallic acid equivalent and flavonoids as quercetin equivalent. Additionally, the estimation of total terpenoids content through the lupeol technique showcased the presence of terpenoids in the extract. The phytochemical analysis by LC-MS revealed the identification of various compounds, including p-Coumaric acid, quinic acid, apigenin, quercetin-3-O-β-D-glucoside, procyanidin B2, β-sitosterol, and others. These compounds contribute to the plant's potential therapeutic properties. Furthermore, the study delved into the cytotoxic effects of the ethanolic extract on SK-MEL-3 skin cancer cells. The MTT assay demonstrated an inhibitory effect, with IC50 values falling within the range of 62.5 to 1000µg/ml. The AO/EB staining technique provided a visual representation of the morphological alterations in the treated cells, indicating potential apoptotic effects induced by the extract. The comprehensive analysis of *C. infundibuliformis* (L.) not only sheds light on its chemical composition but also highlights its potential therapeutic applications, particularly in the realm of cancer treatment. The study sets the stage for further research and exploration of the plant's medicinal properties and opens avenues for the development of novel pharmaceuticals derived from natural sources.

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