INNSPUB

Journal of Biodiversity and Environmental Sciences | JBES
ISSN: 2220-6663 (Print); 2222-3045 (Online)
Website: https://www.innspub.net
E-mail contact: info@innspub.net
Vol. 29, Issue: 1, p. 64-75, 2026

RESEARCH PAPER OPEN ACCESS

GC–MS-based phytochemical profiling and cytotoxic potential of *Cassia auriculata* methanolic leaf extract on human epidermoid carcinoma (A431) cells

V. Vinotha, R. Rajakumar*

[1]*PG and Research Department of Biotechnology, Sir Issc Newton College, Nagapattinam, Affiliated to Bharathidasan University, Tamil Nadu, India*
[2]*PG and Research Department of Biotechnology, Maruthupandiyar College, Thanjavur, Affiliated to Bharathidasan University, Tamil Nadu, India*

Key words: *Cassia auriculata*, Phytochemical screening, TLC, GC–MS, Cytotoxicity, MTT assay, LDH assay, Trypan blue assay, A431 cells, Anticancer activity

Received: 23 June, 2026 **Accepted:** 03 July, 2026 **Published:** 10 July, 2026

DOI: https://dx.doi.org/10.12692/jbes/29.1.64-75

ABSTRACT

Cassia auriculata is a medicinal plant widely recognized in traditional medicine for its diverse therapeutic properties. The present study was undertaken to investigate the phytochemical composition and in vitro cytotoxic potential of the methanolic leaf extract of *Cassia auriculata*. Preliminary qualitative phytochemical screening revealed the presence of alkaloids, flavonoids, phenolics, tannins, saponins, terpenoids, steroids, glycosides, carbohydrates, and proteins. Quantitative analysis demonstrated appreciable levels of total phenolics, flavonoids, and alkaloids, indicating the abundance of antioxidant-rich secondary metabolites. Thin-layer chromatography (TLC) confirmed the presence of alkaloids, flavonoids, and terpenoids with characteristic Rf values, providing a distinct phytochemical fingerprint of the extract. Further characterization by gas chromatography–mass spectrometry (GC–MS) identified twenty-nine bioactive compounds belonging to various chemical classes, including phenolics, fatty acids, terpenoids, carbohydrates, glycosides, alcohols, and esters. Among the identified constituents, 3-O-methyl-D-glucose (48.50%), α-tocopherol-β-D-mannoside (14.22%), resorcinol (11.80%), and n-hexadecanoic acid (3.21%) were the predominant compounds. The cytotoxic activity of the methanolic extract was evaluated against human epidermoid carcinoma cells using MTT, Trypan Blue and Lactate Dehydrogenase assays. The IC_{50} values obtained were 198.6 ± 8.5 µg/ml for the MTT assay, 268.3 ± 10.2 µg/ml for the Trypan Blue assay, and 210.8 ± 9.1 µg/ml for the LDH assay, confirming the ability of the extract to inhibit cell viability and induce membrane damage.

***Corresponding Author:** R. Rajakumar ✉ biotechrajakumar@gmail.com

INTRODUCTION

Medicinal plants have served as an indispensable source of therapeutic agents for centuries and continue to play a pivotal role in global healthcare. According to the World Health Organization (WHO, 2023), a substantial proportion of the world's population, particularly in developing countries, relies on traditional herbal medicines for primary healthcare due to their accessibility, affordability, and perceived safety. Natural products remain an important source of bioactive compounds, contributing to more than half of the drugs currently used in clinical practice, with higher plants accounting for nearly one-quarter of all approved therapeutic agents (Newman and Cragg, 2020). The immense biodiversity of tropical ecosystems provides an invaluable reservoir of phytochemicals with significant pharmacological potential, making medicinal plants promising candidates for the discovery of novel therapeutic agents.

Cancer remains one of the leading causes of morbidity and mortality worldwide and poses a major public health challenge. It is characterized by uncontrolled cellular proliferation resulting from genetic and epigenetic alterations that disrupt normal mechanisms regulating cell growth, differentiation, apoptosis, and DNA repair (Sung *et al.*, 2021). The progression of cancer involves multiple stages, including initiation, promotion, and progression, ultimately leading to local invasion and distant metastasis (Foulds, 1954). Although significant advances have been made in cancer diagnosis and treatment, conventional therapies such as chemotherapy and radiotherapy are frequently associated with severe adverse effects, drug resistance, and limited selectivity toward cancer cells. Consequently, the search for safer and more effective anticancer agents has become a major focus of biomedical research.

Medicinal plants represent an important source of anticancer compounds because of their diverse array of secondary metabolites, including flavonoids, phenolic acids, tannins, alkaloids, terpenoids, and glycosides. These phytochemicals exhibit multiple biological activities, including antioxidant, anti-inflammatory, antiproliferative, and apoptosis-inducing properties, making them attractive candidates for cancer prevention and therapy. More than 60% of currently available anticancer drugs are either directly derived from plants or developed from natural-product scaffolds (Newman and Cragg, 2020). Extensive screening programs conducted by the National Cancer Institute have identified numerous plant extracts with promising anticancer activity; however, many medicinal plants remain inadequately explored for their therapeutic potential (Atanasov *et al.*, 2021; Gahtori *et al.*, 2023).

Among medicinal plants, *Cassia auriculata* L. (Family: Fabaceae, subfamily Caesalpinioideae), commonly known as Tanner's Cassia or Avaram Senna, is widely distributed throughout India and has been extensively used in traditional medicine. Different parts of the plant have been employed for the management of diabetes, liver disorders, skin diseases, ulcers, rheumatism, and microbial infections. Phytochemical investigations have demonstrated that *C. auriculata* is rich in flavonoids, phenolic compounds, tannins, saponins, terpenoids, and glycosides, which contribute to its broad spectrum of pharmacological activities, including antioxidant, antidiabetic, antimicrobial, hepatoprotective, anti-inflammatory, nephroprotective, immunomodulatory, and anticancer effects. Among its various plant parts, the flowers are traditionally consumed as herbal tea for the management of diabetes and are considered an important source of bioactive phytochemicals.

Despite the documented medicinal properties of *C. auriculata*, limited information is available regarding the cytotoxic and antiproliferative potential of its flower extract against skin cancer cells. Therefore, the present study aimed to evaluate the in vitro cytotoxic and antiproliferative activities of *Cassia auriculata* flower extract using MTT, Trypan Blue exclusion, and lactate dehydrogenase (LDH) release assays in a human skin cancer cell line. The findings of this study may provide scientific evidence supporting the development of *C. auriculata* as a potential natural source of anticancer agents.

MATERIALS AND METHODS

Plant material collection and authentication

Fresh leaves of *Cassia auriculata* L. were collected from naturally growing plants in and around Thanjavur, Tamil Nadu, India, during the flowering season. The plant material was authenticated by a qualified taxonomist, and a voucher specimen was deposited in the departmental herbarium for future reference. The collected leaves were washed thoroughly with running tap water followed by distilled water to remove dust and other contaminants. The cleaned leaves were shade-dried at ambient temperature (25–30°C) for 10–15 days until a constant weight was achieved. The dried leaves were pulverized into a fine powder using a laboratory grinder and stored in sterile airtight containers at room temperature until extraction.

Preparation of methanolic leaf extract

The powdered leaf material (1 g) was extracted with 20 mL of analytical-grade methanol (Merck, India) using a rotary shaking incubator at 150 rpm and 40°C for 24 h. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure using a rotary vacuum evaporator at 40°C. The concentrated extract was dried to obtain a crude methanolic extract and stored at 4°C in amber-colored bottles until further phytochemical and biological analyses (Azwanida, 2015).

Preliminary phytochemical screening

The methanolic leaf extract of *C. auriculata* was qualitatively screened for the presence of major classes of phytochemicals, including alkaloids, flavonoids, phenolics, tannins, saponins, terpenoids, steroids, glycosides, quinones, carbohydrates, and proteins using standard phytochemical procedures as described by Ayoola *et al.* (2008) with minor modifications. The appearance of characteristic color changes or precipitates was considered indicative of the presence of the respective phytochemical constituents.

Quantitative phytochemical analysis

Estimation of total phenol

The total phenolic content (TPC) of the methanolic extract of Cassia auriculata was determined spectrophotometrically using the Folin–Ciocalteu method (Singleton and Rossi, 1965). In short, 1 ml of sample was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 10 ml of 7% sodium carbonate solution was added to the mixture, followed by the addition of 13 ml of deionized distilled water and mixed thoroughly. The mixture was kept in dark for 90 min at 23°C. the colour development was read at 750 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing gallic acid solution. The estimation of the phenolic compounds was carried out in triplicates. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per g of dried sample.

Estimation of total flavonoids

Total flavonoid content of the methanolic extracts of *Cassia auriculata* was determined by Park *et al.* (2008). Briefly, to 0.5 ml of extracts, 3.4 ml of 30% methanol was added, followed by 0.15 ml of sodium nitrate (0.5M) and 0.15 ml of aluminium chloride (0.3M) was added. After 5 min, 1 ml of sodium hydroxide (1M) was added and the solution was mixed well and the absorbance was measured against reagent blank at 506 nm. The standard curve for total flavonoids was made using rutin as standard. The total flavonoids were expressed as milligrams of rutin equivalents (RE) per g of dried sample.

Estimation of total alkaloids

The total alkaloid content of the methanolic extracts of *Cassia auriculata* was determined by Harborne (1998) with some modification. In brief, 100 ml of 10% acetic acid in ethanol was added to 1 ml of extracts, filtrated and concentrated on a water bath to 25 ml of its original volume. Then droplets of concentrated ammonium hydroxide were added to the extract until the precipitation of entire solution. Then the solution was allowed to settle and the precipitates were washed with dilute ammonium hydroxide and then filtered using Whatman filter paper. The residue was dried in the oven at 40°C and weighed. Then alkaloid content (%) present in the extracts was determined.

Thin-layer chromatography

Preliminary separation of phytoconstituents was performed using silica gel 60 F254 aluminum-backed TLC plates (Merck, Germany). The methanolic extract was applied as discrete spots using a capillary tube. Plates were developed in an optimized solvent system, air-dried, and visualized under ultraviolet light at 254 and 365 nm. The developed chromatograms were further sprayed with iodine vapor and potassium permanganate ($KMnO_4$) reagent for compound visualization (Harborne, 1998). The retention factor (Rf) values were calculated using the following equation:

Rf = Distance travelled by the compound / Distance travelled by the solvent front

The solvent system providing maximum resolution was selected for subsequent analyses.

Gas chromatography-mass spectrometry analysis

The phytochemical constituents of the methanolic leaf extract were identified using a PerkinElmer Clarus 500 GC–MS system equipped with a BR-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium (99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. Samples were injected in split mode (50:1), with the injector maintained at 280°C and the ion source at 250°C. Electron ionization was performed at 70 eV. Individual compounds were identified by comparing their mass spectra with those available in the NIST/EPA/NIH Mass Spectral Library (NIST 17) (NIST, 2017).

Cell line and cell culture

Human epidermoid carcinoma cells (A431) were obtained from the National Centre for Cell Science. Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), and amphotericin B (5 µg mL^{-1}). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured at 80–90% confluence using TPVG solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in phosphate-buffered saline) and used for experiments between passages 5 and 20.

Preparation of test samples

The dried methanol extract of *Cassia auriculata* leaves was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 1 mg mL^{-1}. The stock solution was sterilized through a 0.22 µm syringe filter and diluted with DMEM containing 2% FBS to obtain the desired concentrations. The final concentration of DMSO in culture medium did not exceed 0.5% (v/v), which served as the vehicle control.

MTT cell viability assay

Cytotoxicity of the methanol extract against A431 cells was evaluated by the MTT assay according to the method of Mosmann (1983) with minor modifications (Denizot and Lang, 1986). Cells were seeded into 96-well plates at a density of 5 × 10^3 cells per well in 100 µL of complete medium and incubated for 24 h to allow attachment. The medium was replaced with fresh medium containing different concentrations of the extract (31.25–500 µg mL^{-1}) and incubated for an additional 24 h. Following treatment, 20 µL of MTT solution (5 mg mL^{-1} in PBS) was added to each well and incubated for 3 h at 37°C. The medium was carefully removed, and the resulting formazan crystals were dissolved in 100 µL DMSO. Absorbance was measured at 570 nm using a microplate reader. Cell growth inhibition was calculated using the following equation:

% of Growth Inhibition = (Control OD- Treated OD/ Control OD) × 100

Trypan blue exclusion assay

Cell membrane integrity was assessed by the Trypan Blue exclusion method (Strober, 2015). A431 cells were seeded into 6-well plates at a density of 1 × 10^4 cells per well and incubated for 24 h. Cells were treated with the methanol extract at concentrations

ranging from 100 to 1000 µg mL^{-1} for 3 h at 37°C. Following treatment, cells were detached with trypsin, collected by centrifugation at 2500 rpm for 5 min, and resuspended in culture medium. An aliquot of cell suspension was mixed with an equal volume of 0.2% Trypan Blue solution and incubated for 3 min. Viable (unstained) and non-viable (blue-stained) cells were counted using a hemocytometer. Percentage cell death was calculated as:

% Dead cells = {(Total cells counted – Total viable cells)/Total cells counted} ×100

Lactate dehydrogenase release assay

Membrane damage induced by the extract was evaluated by measuring extracellular LDH release according to the method of Korzeniewski and Callewaert (1983), with appropriate modifications. A431 cells were seeded into 96-well plates (5×10^3 cells per well) and allowed to attach for 24 h. Cells were treated with different concentrations of the methanolic extract (31.25–500 µg mL^{-1}) for 24 h. After treatment, culture supernatants were collected and mixed with LDH reaction buffer containing Tris-HCl (50 mM, pH 7.4), EDTA (20 mM), sodium dodecyl sulfate (0.5%), pyruvate (1 mM), and NADH (0.2 mM). The decrease in NADH absorbance was monitored at 340 nm using a UV-visible spectrophotometer. Percentage cytotoxicity was calculated relative to untreated control cells.

% of Growth Inhibition = {(Control OD- Treated OD)/ Control OD} × 100

Statistical analysis

All experiments were performed in triplicate (n = 3), and data were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of $p < 0.05$ were considered statistically significant. The half-maximal inhibitory concentration (IC_{50}) values were determined from dose-response curves using nonlinear regression analysis.

RESULTS

Qualitative phytochemical analysis

Phytochemical screening of *Cassia auriculata* demonstrated the presence of several biologically active secondary metabolites. Qualitative analysis of the dried extract revealed that alkaloids, flavonoids, phenolic compounds, tannins, and saponins were present in relatively higher concentrations. In addition, each dried extract was examined for the occurrence of various phytoconstituents, including alkaloids, terpenoids, phenols, tannins, sugars, saponins, flavonoids, quinones, steroids and proteins (Fig. 1). The results of the phytochemical screening are summarized in Table 1.

Fig. 1. Phytochemical analysis of leaves (Methanol)

Table 1. Phytochemical analysis of methanolic leaves extracts of *Cassia auriculata*

Phytochemical tests	Methanol extracts
Alkaloids	+
Terpenoids	-
Phenols and tannins	+
sugar	-
saponins	+
Flavonoids	+
Quinones	+
proteins	-
steroids	-

(+): presence of chemical constituents, (-): absence of chemical constituents

Quantitative phytochemical analysis

Quantitative estimation of the major phytochemical constituents in the methanolic leaf extract of *Cassia auriculata* revealed substantial amounts of phenolic compounds, flavonoids, and alkaloids (Table 2). Among the analyzed constituents, phenolics were the predominant compounds, followed by flavonoids, while alkaloids were detected in moderate quantities. The total phenolic content of the methanolic extract was determined to be 82.46 ± 2.13 mg GAE g^{-1} of dry extract. The abundance of phenolic compounds suggests that the plant possesses significant antioxidant potential, as these metabolites are well recognized for their ability

to neutralize reactive oxygen species and protect biological systems from oxidative damage.

Table 2. Quantitative phytochemical constituents of Cassia auriculata methanolic leaf extract

Phytochemical	Content
Total phenolics	82.46 ± 2.13 mg GAE g^{-1}
Total flavonoids	46.87 ± 1.52 mg RE g^{-1}
Total alkaloids	8.94 ± 0.41 %

The total flavonoid content was found to be 46.87 ± 1.52 mg RE g^{-1} of dry extract. The relatively high flavonoid concentration indicates the presence of important bioactive constituents that may contribute to the antioxidant, anti-inflammatory, antimicrobial, and therapeutic properties of the plant.

The total alkaloid content was estimated at 8.94 ± 0.41% of the dry extract. Although present in lower amounts than phenolics and flavonoids, the alkaloids represent an important class of secondary metabolites with diverse pharmacological activities, including antimicrobial, analgesic, and antidiabetic effects.

Thin layer chromatography (TLC)

Thin-layer chromatography (TLC) analysis was performed to separate and identify the major phytochemical constituents present in the methanolic and aqueous extracts of *Cassia auriculata*.

Table 3. Phytocompounds analysis by TLC plate

Sample	Rf value	Compounds present
Methanol	0.76	Alkaloids
	0.73	Flavonoids
	0.67	Terpenoids
Water	0.76	Alkaloids
Standard	0.71	Ascorbic acid

Distinct bands corresponding to different classes of phytochemicals were observed on the TLC plates, indicating the presence of several bioactive compounds. The methanolic extract exhibited better separation and more intense bands than the aqueous extract, suggesting a higher extraction efficiency for phytoconstituents. The retention factor (Rf) values obtained for the identified compounds were 0.76 for alkaloids, 0.73 for flavonoids, and 0.67 for terpenoids. The Rf value of the standard ascorbic acid was recorded as 0.71, which served as a reference for comparison (Table 3). Among the phytochemical groups analyzed, the alkaloid fraction in the methanolic extract exhibited the most prominent band with the highest Rf value, indicating its relatively greater abundance in the extract (Fig 2). These findings confirm that *Cassia auriculata* leaves contain a diverse range of phytochemicals, with methanol proving to be a more effective solvent for extracting bioactive constituents than water.

Fig. 2. TLC plate showed the various spots
M-Methanol Extract, W-Water Extract, S-Standard

GC-MS of leaves of *Cassisa auriculata* Linn

Gas chromatography–mass spectrometry (GC–MS) profiling of the methanolic leaf extract of *Cassia auriculata* demonstrated a complex mixture of phytochemical constituents distributed across different retention times (Fig. 3, Table 4). The chromatographic analysis identified 29 chemical compounds, representing a broad spectrum of secondary metabolites, including carbohydrates, phenolic compounds, fatty acids, terpenoids, glycosides, esters, aldehydes, alcohols, amines, and other bioactive molecules. The presence of numerous well-defined peaks reflects the chemical diversity of the methanolic extract and indicates the richness of its phytoconstituents.

Among the detected metabolites, 3-O-methyl-D-glucose was the most abundant constituent, accounting for 48.50% of the total peak area with a retention time of 20.14 min. This was followed by α-tocopherol-β-D-mannoside, which contributed 14.22% of the total chromatographic area at 37.23

min, while resorcinol represented 11.80% at 10.73 min. Additional compounds identified in appreciable amounts included n-hexadecanoic acid (3.21%), 13-octadecenal (2.18%), 1,2,3,4-tetrahydroisoquinolin-6-ol-1-carboxylic acid (1.98%), N-acetyltyramine (1.24%), sucrose (1.20%), α-tocopherol (1.16%), and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester (1.00%). Four chromatographic peaks could not be assigned to known compounds using the available spectral database, suggesting the occurrence of unidentified metabolites that warrant further characterization.

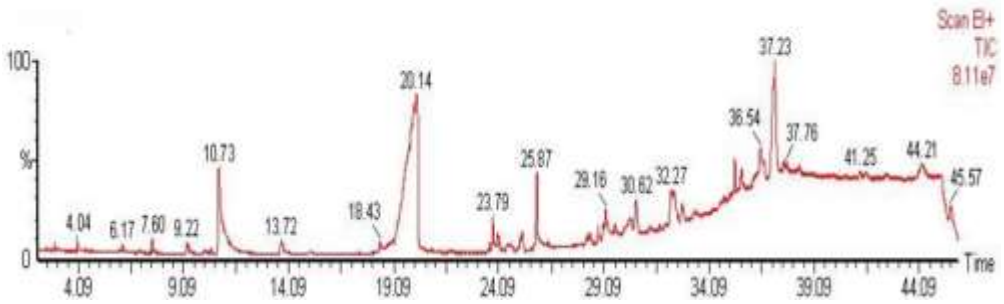

Fig. 3. GC–MS total ion chromatogram of the methanolic leaf extract of *Cassia auriculata*

Table 4. GC-MS analysis of *Cassia auriculata* methanolic leaf extract

Peak no.	RT (min)	Compound name	Molecular formula	Molecular weight (g/mol)	Peak area (%)	Functional group
1	4.04	Glycerin	$C_3H_8O_3$	92	0.16	Triol (Alcohol)
2	6.06	Thymine	$C_5H_6N_2O_2$	126	0.11	Pyrimidine, Amide (Lactam)
3	6.17	1-Butanol, 3-methyl-, formate	$C_6H_{12}O_2$	116	0.17	Ester
4	7.60	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144	0.46	Pyranone, Phenolic hydroxyl
5	9.22	Benzaldehyde, 4-methyl-	C_8H_8O	120	0.83	Aromatic aldehyde
6	10.38	2-Propenoic acid, 4-methylpentyl ester	$C_9H_{16}O_2$	156	0.12	Ester
7	10.73	Resorcinol	$C_6H_6O_2$	110	11.80	Phenol (Dihydroxybenzene)
8	13.72	Sucrose	$C_{12}H_{22}O_{11}$	342	1.20	Carbohydrate (Disaccharide)
9	15.07	1,6-Anhydro-β-D-glucopyranose (Levoglucosan)	$C_6H_{10}O_5$	162	0.30	Carbohydrate
10	18.43	β-D-Glucopyranoside, methyl	$C_7H_{14}O_6$	194	0.36	Glycoside
11	20.14	3-O-Methyl-D-glucose	$C_7H_{14}O_6$	194	48.50	Carbohydrate (Methylated sugar)
12	23.79	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278	1.00	Phthalate ester
13	24.04	Benzenamine, 2,3,4,5,6-pentamethyl	$C_{11}H_{17}N$	163	0.87	Aromatic amine
14	25.20	Unknown	—	—	0.57	Not identified
15	25.87	n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256	3.21	Saturated fatty acid
16	26.42	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	0.10	Fatty acid ester
17	28.29	1-Tridecyne	$C_{13}H_{24}$	180	0.30	Alkyne
18	28.41	13-Oxabicyclo[10.1.0]tridecane	$C_{12}H_{22}O$	182	0.42	Cyclic ether
19	28.80	Phytol	$C_{20}H_{40}O$	296	0.61	Diterpene alcohol
20	29.06	1-E,11-Z-13-Octadecatriene	$C_{18}H_{32}$	248	0.56	Unsaturated hydrocarbon
21	29.16	13-Octadecenal, (Z)-	$C_{18}H_{34}O$	266	2.18	Aldehyde
22	29.58	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	284	0.46	Saturated fatty acid
23	30.62	1,2,3,4-Tetrahydroisoquinolin-6-ol-1-carboxylic acid	$C_{11}H_{13}NO_3$*	207	1.98	Alkaloid, Phenol, Carboxylic acid
24	32.26	Unknown	—	—	3.29	Not identified
25	32.44	Unknown	—	—	2.61	Not identified
26	32.82	α-Tocopherol (Vitamin E)	$C_{28}H_{48}O_2$	416	1.16	Phenolic antioxidant
27	35.28	N-Acetyltyramine	$C_{10}H_{13}NO_2$	179	1.24	Amide, Phenol
28	35.60	Unknown	—	—	1.14	Not identified
29	37.23	α-Tocopherol-β-D-mannoside	$C_{35}H_{60}O_7$	592	14.22	Glycoside, Phenolic antioxidant

The identified compounds belong to several pharmacologically important chemical groups that are known to contribute to the biological activities of medicinal plants. Phenolic constituents, including resorcinol and α-tocopherol, are well documented for their antioxidant properties and their ability to protect cells from oxidative stress. Fatty acids such as n-hexadecanoic acid and octadecanoic acid have been reported to exhibit antimicrobial, anti-inflammatory, and membrane-stabilizing effects. The diterpenoid alcohol phytol has been associated with antioxidant, anticancer, antimicrobial, and immunomodulatory activities. Furthermore, the presence of carbohydrates and glycosides may enhance the nutritional value and therapeutic efficacy of the extract by serving as precursors or carriers of biologically active compounds.

Cytotoxicity assessment by MTT, trypan Blue and LDH assays

The cytotoxic activity of the methanolic leaf extract of *Cassia auriculata* was evaluated using three complementary assays, namely MTT, Trypan Blue (TB) exclusion, and Lactate Dehydrogenase (LDH) release assays. The extract exhibited a concentration-dependent increase in cytotoxicity across all three methods, indicating its ability to inhibit cell viability and induce cellular damage (Fig. 4).

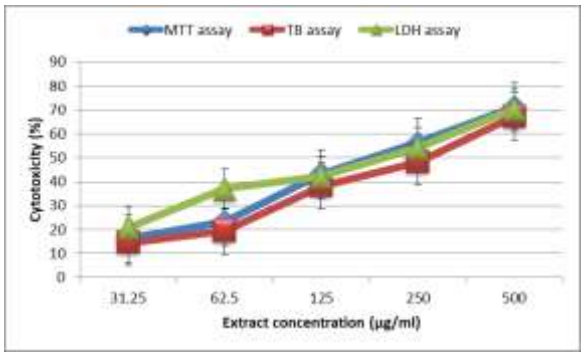

Fig. 4. *In vitro* cytotoxic effects of the methanolic leaf extract of *Cassia auriculata* at different concentrations as assessed by MTT, Trypan Blue, and LDH assays.

MTT assay

The cytotoxic activity of the methanolic leaf extract of *Cassia auriculata* against human epidermoid carcinoma (A431) cells was evaluated using the MTT assay, which measures mitochondrial metabolic activity as an indicator of cell viability. The extract exhibited a concentration-dependent inhibitory effect on A431 cell proliferation. At the lowest concentration (31.25 µg/ml), the extract induced 16.21 ± 0.72% cytotoxicity. The percentage of cytotoxicity increased progressively to 23.45 ± 0.91%, 43.26 ± 1.24%, 56.32 ± 1.51%, and 71.24 ± 1.86% at concentrations of 62.50, 125.00, 250.00, and 500.00 µg/ml, respectively. The IC_{50} value was calculated as 198.6 ± 8.5 µg/ml, indicating that the methanolic extract effectively reduced the viability of A431 cells in a dose-dependent manner. These findings suggest that the bioactive constituents present in the extract possess significant antiproliferative activity against human epidermoid carcinoma cells.

Trypan blue exclusion assay

The cytotoxic potential of the methanolic leaf extract against A431 human epidermoid carcinoma cells was further assessed using the Trypan Blue exclusion assay, which differentiates viable cells from non-viable cells based on membrane integrity. A concentration-dependent increase in cell death was observed following treatment with the extract. The percentage of cytotoxicity increased from 14.44 ± 0.68% at 31.25 µg/ml to 19.35 ± 0.84%, 38.24 ± 1.17%, 48.06 ± 1.42%, and 67.29 ± 1.73% at 62.50, 125.00, 250.00, and 500.00 µg/ml, respectively. The calculated IC_{50} value was 268.3 ± 10.2 µg/ml, indicating moderate cytotoxic efficacy against A431 cells. The results demonstrate that the methanolic extract significantly compromises cell viability in a concentration-dependent manner.

Table 5. IC_{50} values of cytotoxic activity of methanolic leaf extract of *Cassia auriculata* against A431 cell line in various assay

Assay	Cytotoxicity activity IC50 (µg/mL)
MTT assay	198.6± 8.5
TB assay	268.3± 10.2
LDH assay	210.8±9.1

Lactate dehydrogenase release assay

The membrane-damaging effect of the methanolic leaf extract on human epidermoid carcinoma (A431) cells was investigated using the Lactate Dehydrogenase (LDH) release assay. LDH is a cytoplasmic enzyme that is released into the culture medium when the plasma membrane is damaged, making it a reliable marker of cytotoxicity. As shown in Fig. 5, LDH release increased progressively with increasing concentrations of the extract. The percentage of cytotoxicity was 20.88 ± 0.81% at 31.25 µg/ml, increasing to 37.09 ± 1.02%, 42.40 ± 1.28%, 54.28 ± 1.49%, and 70.50 ± 1.82% at 62.50, 125.00, 250.00, and 500.00 µg/ml, respectively. The IC_{50} value obtained from the LDH assay was 210.8 ± 9.1 µg/ml, demonstrating that the methanolic extract caused substantial membrane damage to A431 cells in a concentration-dependent manner (Table 5).

DISCUSSION

The present investigation demonstrated that the methanolic leaf extract of *Cassia auriculata* contains a wide spectrum of bioactive secondary metabolites. Qualitative phytochemical screening confirmed the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, terpenoids, steroids, carbohydrates, proteins, and glycosides. Among these, phenolics, flavonoids, alkaloids, tannins, and saponins were the predominant constituents, indicating that the plant is a rich reservoir of naturally occurring phytochemicals. The occurrence of these metabolites substantiates the traditional medicinal importance of *C. auriculata*, which has long been employed in the treatment of diabetes, inflammatory disorders, microbial infections, and other ailments associated with oxidative stress (Harborne, 1998; Kirtikar and Basu, 2005).

Quantitative analysis further revealed considerable amounts of total phenolics, flavonoids, and alkaloids in the methanolic extract. Phenolic compounds are among the most effective natural antioxidants owing to their ability to donate electrons or hydrogen atoms to neutralize reactive oxygen species, thereby protecting biological molecules from oxidative injury (Rice-Evans *et al.*, 1997). Likewise, flavonoids have been extensively reported to exhibit antioxidant, antimicrobial, anti-inflammatory, and anticancer properties through modulation of oxidative stress and intracellular signaling pathways (Panche *et al.*, 2016). Alkaloids also represent an important group of secondary metabolites with documented antimicrobial, analgesic, antidiabetic, and anticancer activities (Harborne, 1998). Therefore, the therapeutic efficacy of *C. auriculata* observed in the present study may result from the combined action of these phytochemicals rather than from a single active constituent. These observations are consistent with earlier reports describing *C. auriculata* as an excellent source of polyphenols, flavonoids, tannins, and alkaloids, which collectively contribute to its antioxidant and medicinal properties (Anushia *et al.*, 2009).

Thin-layer chromatography was employed as a preliminary analytical technique to characterize the phytochemical composition of the methanolic leaf extract. The development of well-defined chromatographic bands with characteristic retention factor (Rf) values confirmed the presence of multiple classes of secondary metabolites. Compared with the aqueous extract, the methanolic extract produced more distinct and intense bands, indicating the superior extraction efficiency of methanol for isolating phytochemicals from plant tissues (Harborne, 1998). The Rf values obtained for alkaloids (0.76), flavonoids (0.73), and terpenoids (0.67) closely corresponded with their expected chromatographic behavior, whereas ascorbic acid exhibited an Rf value of 0.71 and served as the reference standard. The pronounced alkaloid band suggested that alkaloids were present in relatively higher concentrations than the other phytochemical groups. TLC is recognized as a rapid, economical, and dependable analytical method for phytochemical fingerprinting and is widely used for the preliminary identification of plant metabolites prior to detailed spectroscopic characterization (Wagner and Bladt, 1996). The chromatographic pattern observed in this study supports the results of the qualitative phytochemical screening and confirms the chemical diversity of the methanolic extract.

Comprehensive characterization of the methanolic extract using GC–MS revealed the presence of twenty-nine phytochemical constituents representing different classes of naturally occurring compounds, including carbohydrates, phenolics, fatty acids, terpenoids, glycosides, esters, aldehydes, alcohols, and aromatic compounds. The occurrence of these chemically diverse metabolites provides a scientific basis for the wide range of biological activities attributed to *Cassia auriculata* (Parekh and Chanda, 2007).

Among the identified compounds, 3-O-methyl-D-glucose constituted the major component (48.50%), followed by α-tocopherol-β-D-mannoside (14.22%), resorcinol (11.80%), and n-hexadecanoic acid (3.21%). The presence of resorcinol and tocopherol

derivatives is particularly significant because these compounds possess strong antioxidant properties capable of protecting cells against oxidative damage by scavenging free radicals (Rice-Evans *et al.*, 1997). Similarly, fatty acids such as n-hexadecanoic acid and octadecanoic acid have been reported to exhibit antimicrobial, anti-inflammatory, antioxidant, and membrane-protective activities. The diterpene alcohol phytol, also identified in the extract, has been associated with antioxidant, anticancer, antimicrobial, and immunomodulatory effects (de Moraes *et al.*, 2014). Collectively, the identified phytochemicals may act synergistically to enhance the pharmacological potential of the plant extract. The GC–MS profile obtained in the present study therefore provides substantial chemical evidence supporting the antioxidant and therapeutic properties of *C. auriculata*. The coexistence of phenolic compounds, fatty acids, terpenoids, and glycosides may explain the diverse biological activities exhibited by the methanolic extract.

The cytotoxic activity of the methanolic leaf extract of *Cassia auriculata* was assessed against human epidermoid carcinoma (A431) cells using MTT, Trypan Blue exclusion, and LDH release assays. All three assays consistently demonstrated a concentration-dependent increase in cytotoxicity, indicating that the extract effectively inhibited the proliferation and survival of A431 cells. Among the three methods, the MTT assay showed the greatest sensitivity, with an IC_{50} value of 198.6 ± 8.5 µg/ml, reflecting a marked reduction in mitochondrial metabolic activity following treatment (Mosmann, 1983). The Trypan Blue exclusion assay produced an IC_{50} value of 268.3 ± 10.2 µg/ml, indicating progressive loss of cell viability as the extract concentration increased. Likewise, the LDH release assay yielded an IC_{50} value of 210.8 ± 9.1 µg/ml, demonstrating significant membrane damage due to the leakage of intracellular LDH into the culture medium (Korzeniewski and Callewaert, 1983). The consistency among these independent assays confirms that the methanolic extract exerts a pronounced cytotoxic effect against A431 cells.

The observed cytotoxic activity may be attributed to the combined action of the bioactive compounds identified through phytochemical and GC–MS analyses. Phenolic compounds and flavonoids are known to induce apoptosis by regulating oxidative stress and activating intracellular signaling pathways involved in programmed cell death. Terpenoids and alkaloids have also been reported to inhibit cancer cell proliferation by arresting the cell cycle and promoting apoptosis, whereas phytol and tocopherol derivatives possess antioxidant and antiproliferative properties that further contribute to cancer cell inhibition (Panche *et al.*, 2016; de Moraes *et al.*, 2014). The synergistic interaction of these metabolites is likely responsible for the significant cytotoxic response observed in the present investigation.

Overall, the relatively low IC_{50} values obtained from the MTT, Trypan Blue, and LDH assays indicate that the methanolic leaf extract of *Cassia auriculata* possesses considerable anticancer potential against human epidermoid carcinoma cells. Nevertheless, further studies involving apoptosis assays, flow cytometric analysis, molecular signaling pathways, and *in vivo* experimental models are essential to validate these findings and to elucidate the precise mechanisms responsible for its anticancer activity.

CONCLUSION

The present study demonstrated that the methanolic leaf extract of *Cassia auriculata* is a rich source of bioactive phytochemicals, including phenolics, flavonoids, alkaloids, tannins, saponins, and terpenoids. TLC and GC–MS analyses confirmed the presence of several biologically active compounds, with 3-O-methyl-D-glucose, α-tocopherol-β-D-mannoside, resorcinol, and n-hexadecanoic acid identified as the major constituents. The extract exhibited significant concentration-dependent cytotoxic activity against human epidermoid carcinoma cells, as demonstrated by MTT, Trypan Blue, and LDH assays. These findings provide scientific evidence supporting the traditional medicinal value of *Cassia auriculata* and highlight its potential as a promising natural source of antioxidant and anticancer agents. Further studies are warranted to isolate the active compounds and elucidate their molecular mechanisms of action.

ACKNOWLEDGEMENTS

The authors wish to thanks the Research Department of Biotechnology, Maruthupandiyar College, Thanjavur for providing necessary facilities that successful completion of the work.

REFERENCES

Anushia C, Sampathkumar P, Ramkumar L. 2009. Antibacterial and antioxidant activities of *Cassia auriculata*. Global Journal of Pharmacology **3(3)**, 127–130.

Atanasov AG, Zotchev SB, Dirsch VM, International Natural Product Sciences Taskforce, Supuran CT. 2021. Natural products in drug discovery: Advances and opportunities. Nature Reviews Drug Discovery **20**, 200–216.
DOI: 10.1038/s41573-020-00114-z

Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO. 2008. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in southwestern Nigeria. Tropical Journal of Pharmaceutical Research **7(3)**, 1019–1024.
DOI: 10.4314/tjpr.v7i3.14686

Azwanida NN. 2015. A review on the extraction methods used in medicinal plants. Medicinal and Aromatic Plants **4(3)**, 196.

Denizot F, Lang R. 1986. Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods **89(2)**, 271–277.
DOI: 10.1016/0022-1759(86)90368-6

De Moraes J, de Oliveira RN, Costa JP, Junior ALG, de Sousa DP, Freitas RM, Allegretti SM, Pinto PLS. 2014. Phytol, a diterpene alcohol from chlorophyll, as a drug against neglected tropical disease schistosomiasis mansoni. PLoS Neglected Tropical Diseases **8(1)**, e2617.
DOI: 10.1371/journal.pntd.0002617.

Foulds L. 1954. The experimental study of tumor progression: A review. Cancer Research **14(5)**, 327–339.

Gahtori R, Tripathi AH, Kumari A, Negi N, Paliwal A, Tripathi P, Joshi P, Rai RC, Upadhyay SK. 2023. Anticancer plant-derivatives: Deciphering their oncopreventive and therapeutic potential in molecular terms. Future Journal of Pharmaceutical Sciences **9**, 14.
DOI: 10.1186/s43094-023-00465-5

Harborne JB. 1998. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd ed. Chapman and Hall.

Kirtikar KR, Basu BD. 2005. Indian Medicinal Plants. 2nd ed. International Book Distributors.

Korzeniewski C, Callewaert DM. 1983. An enzyme-release assay for natural cytotoxicity. Journal of Immunological Methods 64(3), 313–320.
DOI: 10.1016/0022-1759(83)90438-6.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods **65(1–2)**, 55–63.

National Institute of Standards and Technology (NIST). 2017. NIST/EPA/NIH Mass Spectral Library (NIST 17) and NIST Mass Spectral Search Program, Version 2.3. Standard Reference Database 1A. National Institute of Standards and Technology, Gaithersburg, MD, USA.

Newman DJ, Cragg GM. 2020. Natural products as sources of new drugs over the nearly four decades from 1981 to 2019. Journal of Natural Products **83(3)**, 770–803.

Panche AN, Diwan AD, Chandra SR. 2016. Flavonoids: An overview. Journal of Nutritional Science **5**, e47.

Parekh J, Chanda S. 2007. *In vitro* antimicrobial activity and phytochemical analysis of medicinal plants. Turkish Journal of Biology **31**, 53–58.

Park YS, Jung ST, Kang SG, Heo BG, Arancibia-Avila P, Toledo F, Drzewiecki J, Namiesnik J, Gorinstein S. 2008. Antioxidants and proteins in ethylene-treated kiwifruits. Food Chemistry 107(2), 640–648.
DOI: 10.1016/j.foodchem.2007.08.070

Rice-Evans CA, Miller NJ, Paganga G. 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science **2(4)**, 152–159.

Singleton VL, Rossi JA Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture **16(3)**, 144–158.
DOI: 10.5344/ajev.1965.16.3.144

Strober W. 2015. Trypan blue exclusion test of cell viability. Current Protocols in Immunology **111**, A3.B.1-A3.B.3.
DOI: 10.1002/0471142735.ima03bs111

Sung H, Ferlay J, Siegel RL. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians **71(3)**, 209–249.
DOI: 10.3322/caac.21660

Wagner H, Bladt S. 1996. Plant Drug Analysis: A Thin Layer Chromatography Atlas. 2nd ed. Springer.

World Health Organization. 2023. Traditional, Complementary and Integrative Medicine. World Health Organization.