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Multifaceted Anticancer Potential of *Gnidia glauca* (Fresen.) Gilg Leaf Alkaloids: Impact on Multiple Cellular Targets

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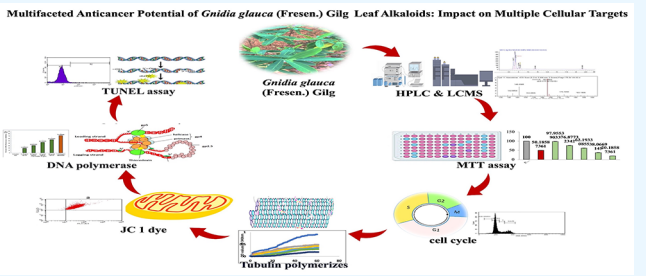
ABSTRACT: *Gnidia glauca* (Fresen.) Gilg has demonstrated significant anticancer potential through multiple mechanisms, including apoptosis induction, as shown by the TUNEL assay against MCF-7 cells, modulation of tubulin polymerization, preservation of mitochondrial function indicated by the JC-1 assay, and inhibition of DNA polymerase α and β activities. Rationale for the present study is to investigate the potential anticancer properties of *G. glauca* leaf alkaloid extract. Fresh and healthy *G. glauca* leaves were cleaned, shade-dried, and the powder was defatted, extracted with 10% acetic acid in ethanol, and subjected for alkaloid extraction. The partially purified *G. glauca* leaf alkaloid extract was evaluated for its effects on tubulin polymerization, DNA polymerase activity, mitochondrial membrane potential, and apoptosis studies using human breast cancer (MCF-7) cells by flow cytometry. The extract was found to affect microtubule assembly in a concentration-dependent manner (15.125−250 μg/mL), indicating presence of alkaloids that function as spindle poison agents. Leaf alkaloid extract of *G. glauca* was also found to affect the mitochondrial membrane potential with IC_{50} value 144.51 μg/mL, and inhibited DNA polymerase α and β activities dose dependently, thus potentially interfering with DNA replication and repair processes. Leaf alkaloid extract also showed the potential to induce DNA damage of 53.6%, albeit somewhat less than the standard drug camptothecin (64.94%) as confirmed by the TUNEL assay. Additionally, the GgLAE (IC_{50} 144.51 μg/mL) showed significant inhibition of MCF-7 cells proliferation after 24 h, revealing phase arrests in sub G0/G1, S, and G2/M. These findings suggest that *G. glauca* leaf alkaloid extract contains alkaloids that possess anticancer properties with multiple targets, making the plant a natural source for a promising phytochemical drug candidates for further evaluation in pre-clinical and clinical studies. Further investigations are warranted to determine the efficacy, safety, identification and characterization of the alkaloids, and evaluate and determine their potential applications in cancer therapy.



1. INTRODUCTION

Gnidia glauca (Fresen.) Gilg, also known as "rameta" or "datpadi," is a therapeutic medicinal plant commonly found in various regions of Africa, India, and other parts of Asia. It has been traditionally used in Ayurveda and African folk medicine to treat several human ailments including fever, malaria, inflammation, and pain.[1] The plant consists of phytochemicals, including alkaloids, terpenoids, flavonoids, and phenolic compounds, which contribute to its pharmacological activities. Among these compounds, alkaloids are recognized as the primary bioactive constituents of the plant. Alkaloids, naturally occurring nitrogenous compounds in plants, have undergone substantial research to determine their various pharmacological characteristics. Phytochemical analysis of *G. glauca* leaf extracts confirms the presence of alkaloids.[2] *G. glauca* has yielded several isolated alkaloids, such as gnidimacrin, gnidicin, and gnididione, demonstrating anticancer, antimalarial, anti-inflammatory, and antioxidant effects.[3] Indole alkaloids have been evaluated and shown to possess anticancer properties, which target the cell cycle processes, including spindle poisons, DNA polymerase inhibitors, and DNA topoisomerase inhibitors (Vinca alkaloids). In the present study, it is proposed to evaluate the anticancer potential of *G. glauca* leaf alkaloid extract (GgLAE) by different assays, used for the evaluation of cytotoxicity of phytochemical constituents, such as MTT assay for cytotoxicity, tubulin polymerization inhibition (spindle poison) assay, DNA polymerase α and β inhibition assays, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay for the apoptotic death confirmation, and JC-1 assay for membrane potential alteration. Moreover, conducting investigations pointing on multiple targets

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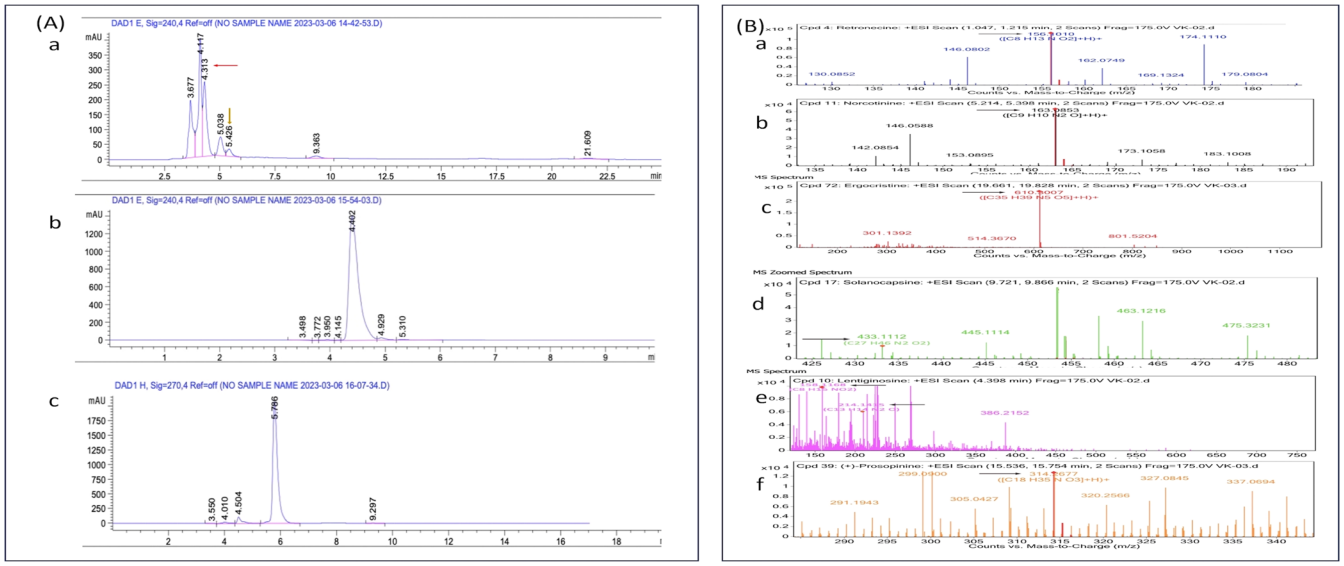

Figure 1. (A) RP-HPLC and LC-HRMS analyses of *G. glauca* alkaloid extract (GgLAE): (a) RP-HPLC chromatogram of *G. glauca* leaf alkaloid extract (GgLAE), (b) RP-HPLC chromatogram of caffeine standard (retention time: 4.402), and (c) RP-HPLC chromatogram of colchicine standard (retention time: 5.786). (B) LC-HRMS of *G. glauca* leaf alkaloid extract for the identification of alkaloids: (a) retronecine, (b) norcotinine, (c) ergocristine, and (d) solanocapsine.

enhances the possibilities of exploration of the pharmacological activities of *G. glauca*. By examining the alkaloid extract of *G. glauca*, it has been demonstrated to have strong and potent cytotoxicity against cancer cells. Its capacity to prevent tubulin polymerization is thought to be its mode of action, besides inducing mitochondrial depolarization, inhibiting DNA polymerase activity, and activating apoptosis. These findings suggest *G. glauca*'s potential as a source of bioactive chemicals for developing new anticancer medicines. Exploring the pharmacological potential of *G. glauca* and its alkaloid extract requires more research.

The cell cycle, governing cell growth and division plays a crucial role in maintaining normal cellular functions. Investigating compounds like *G. glauca* leaf alkaloid extract (GgLAE) provides insights into potential therapeutic strategies by understanding their impact on the cell cycle. Such studies contribute to advancing cancer treatment by targeting aberrant cell division processes.

Tubulin is a major constituent of microtubules and essential cytoskeletal component of eukaryotic cells, which is involved in several cellular processes such as cell division, intracellular transport, and cellular architecture. Tubulin polymerization is a critical process that regulates microtubule assembly and disassembly. Several anticancer drugs such as Taxol and Vinca alkaloids target tubulin polymerization, disrupting microtubule dynamics and cell cycle arrest.

Some of the phytochemicals have been shown to modulate the mitochondrial membrane potential. Quercetin is a flavonoid that is prevalent in fruits and vegetables and has been found to protect mitochondrial function by maintaining membrane potential.[4] JC-1 assay is a common technique used to study the potential of the mitochondrial membrane, an essential parameter of mitochondrial activity. Mitochondrial dysfunction is implicated in several diseases including cancer, neurodegenerative diseases, and metabolic disorders. Many natural products have been shown to have promising effects on mitochondrial function, making them potential candidates for modulating mitochondrial membrane potential, as assessed by the JC-1 assay.

DNA polymerase α and β enzymes are crucial for DNA replication and have been targeted by various natural products that inhibit cell proliferation. *In vitro* studies have demonstrated that crude plant extracts of *Baeckea gunniana* effectively inhibit DNA polymerase β activity, leading to the suppression of cancer cell growth.[5] Similarly, the inhibition of DNA polymerase α activity has been observed in *Arabidopsis*, resulting in the inhibition of cancer cell proliferation.[6] These examples highlight the potential of natural products in modulating DNA polymerases activities as a strategy for combating cancer.

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay is a widely employed technique for understanding the detection of apoptotic cells and evaluation of apoptotic potential of anticancer compounds. TUNEL assay is utilized to detect DNA fragmentation as a hallmark of apoptosis. This assay has also been utilized to evaluate the apoptotic effects of various natural products, including extracts from medicinal plants. For instance, an extract derived from *Morus alba* (white mulberry) leaves demonstrated apoptotic effects on colorectal cancer cells, as confirmed by the TUNEL assay. Ethanol extract from *Paeonia lactiflora* (peony) roots induced apoptosis in human leukemia cells.[7] These findings underscore the utility of the TUNEL assay in assessing the apoptotic potential of natural products in different cell types and provide insights into their mechanisms of action. In view of the anticancer properties of phytochemicals and availability of standardized confirmation procedures for the evaluation of anticancer activity, the present study has been carried out to evaluate the anticancer properties of *G. glauca* leaf alkaloid extract.

2. RESULTS AND DISCUSSION

2.1. RP-HPLC Analysis of GgLAE. To separate and identify the alkaloids present in *G. glauca* leaf alkaloid extract, reverse-phase high-performance liquid chromatography (RP-

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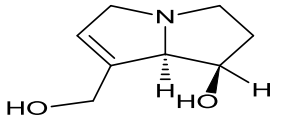
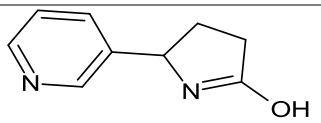
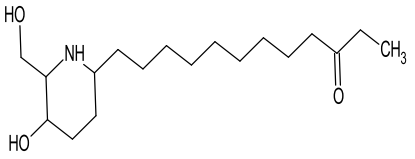
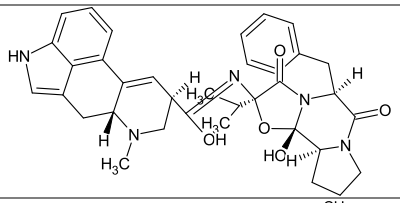
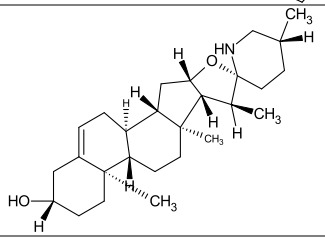
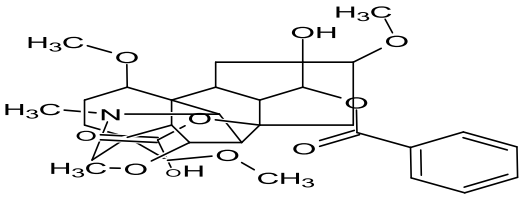
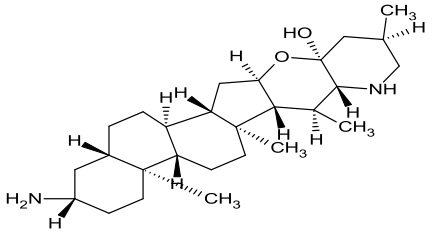
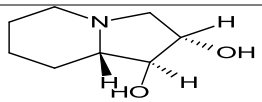
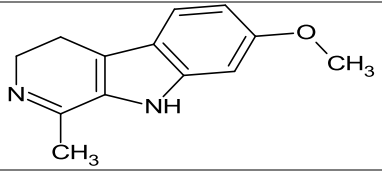
Table 1. Identified Compounds Present in *G. glauca* Leaf Alkaloid Extract by LC-HRMS

Sl. No.	Rt	Compound name	Structure	Molecular formula	m/z
1	1.172	Retronecine		$C_8H_{13}NO_2$	156.1009
2	5.176	Norcotinine		$C_9H_{10}N_2O$	163.0851
3	15.677	(+)-Prosopinine		$C_{18}H_{35}NO_3$	314.2677
4	19.759	Ergocristine		$C_{35}H_{39}N_5O_5$	610.3007
5	24.327	Solasodine		$C_{27}H_{43}NO_2$	413.2636
6	24.887	Delphinine		$C_{33}H_{45}NO_9$	598.59
7	10.133	Solanocapsine		$C_{27}H_{46}N_2O_2$	433.1118
8	4.419	Lentiginosine		$C_8H_{15}NO_2$	158.1168
9	4.419	Harmidine		$C_{13}H_{14}N_2O$	214.1415

HPLC) was performed. Results revealed the presence of six metabolites exhibiting distinct retention times: 3.677, 4.177, 4.313, 5.038, 5.428, and 9.36 min, respectively (Figure 1A). Presence of alkaloids in GgLAE was confirmed with the help of standards caffeine (Rt, 4.402) and colchicine (Rt, 5.786) (Figure 1A[b,c]). Caffeine is an alkaloid and a well-known

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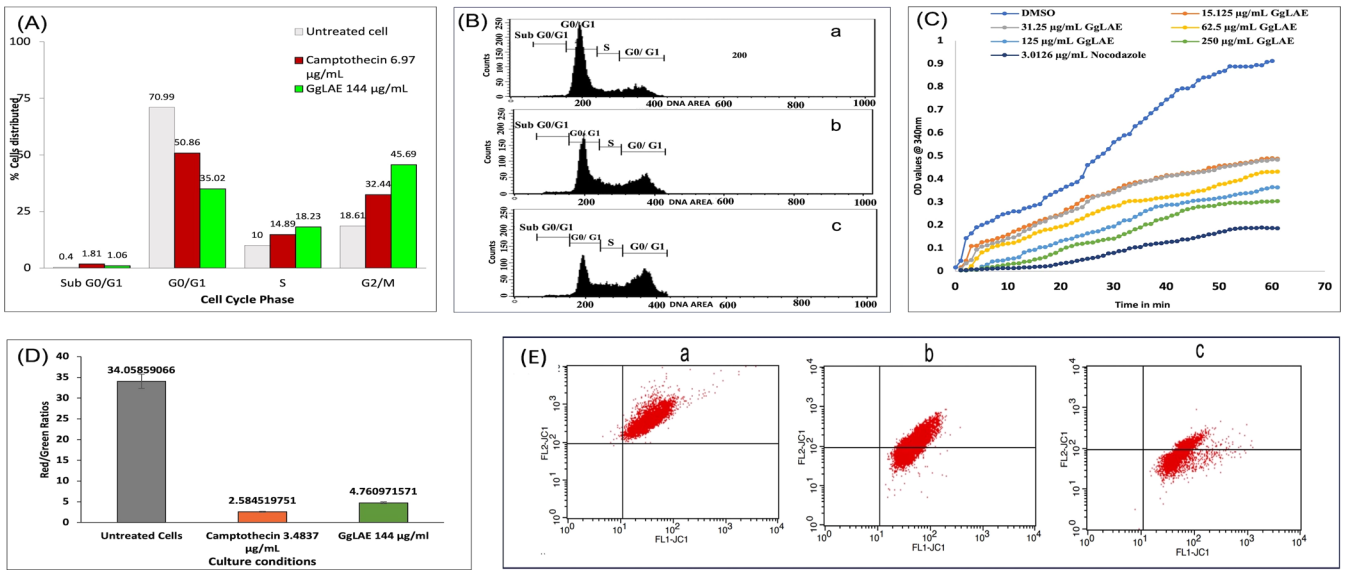

Figure 2. (A, B) Overlaid bar graph showing the % of cells getting arrested or distributed in the different phases of MCF-7 cell cycle upon treatment with camptothecin and GgLAE in comparison to the control. (C) Assessment of anticancer property of GgLAE in MCF-7 cells by tubulin polymerization inhibition by GgLAE: Mean OD values of tubulins treated with GgLAE at different concentrations, and standard nocodazole. (D) JC-1 assay for mitochondrial membrane potential changes induced by GgLAE. (E) JC-1 expression in MCF-7 cells post 24-h incubation with (a) untreated cells, (b) GgLAE, and (c) camptothecin. UR (%): high mitochondrial membrane potential (healthy cells); LR (%): low mitochondrial membrane potential (unhealthy cells). FL1 (*X*-axis): green fluorescence, FL2 (*Y*-axis): red fluorescence.

central nervous system stimulant,[22] while colchicine is an alkaloid used as an anti-inflammatory and anticancer agent that interferes with the formation of microtubules.[23]

2.2. Liquid Chromatography−High Resolution Mass Spectrometry (LC-HRMS). Liquid chromatography−high-resolution mass spectrometry (LC-HRMS) analysis of GgLAE suggests the presence of ten distinct compounds (Figure 1B). These compounds were identified by their retention times (Rt) and fragments' mass (m/z) values (Table 1), which include several well-known alkaloids such as retronecine, norcotinine, solasodine, and delphinine. Retronecine and norcotinine are pyrrolizidine alkaloids, which are known for their toxicity.[24] Solasodine is a steroidal alkaloid with known anticancer and anti-inflammatory properties.[25] Delphinine is a diterpene alkaloid that has been reported to possess antitumor activity.[26,27]

2.3. Cytotoxicity Assessment by MTT Assay. Mosmann originally developed the MTT assay in 1983 to measure cell viability and cytotoxicity. Researchers have recently been evaluating plant extract alkaloids using the MTT assay due to their potential therapeutic properties.[28] Anticancer activity of GgLAE (*G. glauca* leaf alkaloid extract) was evaluated by assessing its cytotoxicity through the MTT assay in MCF-7 (IC_{50} of 144.51 μg/mL) and MCF-10A (IC_{50} of 409.86 μg/mL) cell lines. Similarly, the partially purified *G. glauca* leaf alkaloid fraction (GgLAF) has been evaluated for cytotoxicity by MTT assay in MCF-7 (IC_{50} of 69.04 μg/mL), HT-29 (IC_{50} of 36.34 μg/mL), and HeLa (IC_{50} of 47.27 μg/mL). GgLAE demonstrated a concentration-dependent decrease in cell viability that was caused by different concentrations of GgLAE (12.5−200 μg/mL) after the 24 h of incubation (data not shown as depiction). Camptothecin (IC_{50} of 6.97 μg/mL) used as a positive control demonstrated a comparable dose-dependent impact on cell viability. These findings highlight the broad-spectrum cytotoxic potential of GgLAE across different cancer cell types.

2.4. Cell Cycle Analysis Using Propidium Iodide. In this study, the GgLAE demonstrated significant cell inhibition against MCF-7 cell lines after a 24 h treatment period. The IC_{50} concentration 144.51 μg/mL was selected for further investigation. Cell cycle analysis was conducted by flow cytometry, and the results are depicted (Figure 2A,B). The percentages of cells arrested in different phases of the MCF-7 cell cycle were assessed. In the sub G0/G1 phase (apoptotic phase), 0.4, 1.81, and 1.06% of cells were arrested in the untreated, camptothecin (10 μM), and GgLAE (144 μg/mL) groups, respectively. For the G0/G1 phase (growth phase), 70.99, 50.86, and 35.02% of cells were arrested in the untreated, camptothecin, and GgLAE groups, respectively. In the S phase (synthetic phase), 10, 14.89, and 18.23% of cells were arrested in the untreated, camptothecin, and GgLAE groups, respectively. Conversely, in the G2/M phase, 18.61, 32.44, and 45.69% of cells were arrested in the untreated, camptothecin, and GgLAE groups, respectively. The MCF-7 cells treated with GgLAE at the IC_{50} concentration exhibited an increased percentage of cells at G2/M and S phases while inhibiting the percentage of cells at the G0/G1 phase, similar to camptothecin. This indicates that the GgLAE arrests the cell cycle at G2/M and S phases, suggesting its potential as a potent antibreast cancer drug, inhibiting cell division and proliferation (Figure 2A,B).

The cell cycle study reinforced findings from prior research, validating observed patterns of *Hemizonia floribunda* induced significant cell cycle changes in HT-29, HeLa, and MCF-7 cells. Some natural anticancer drugs (paclitaxel, docetaxel, vinblastine, vincristine) act on the G2/M phase. G0/G1 and G2/M arrests may crucially contribute to cytotoxicity, offering avenues for future therapeutic exploration.[29]

2.5. Tubulin Polymerization Assay. Results of the tubulin polymerization assay through GgLAE showed that tubulin polymerization was inhibited by GgLAE in a dosage-dependent and time-based manner (Figure 2C), suggesting

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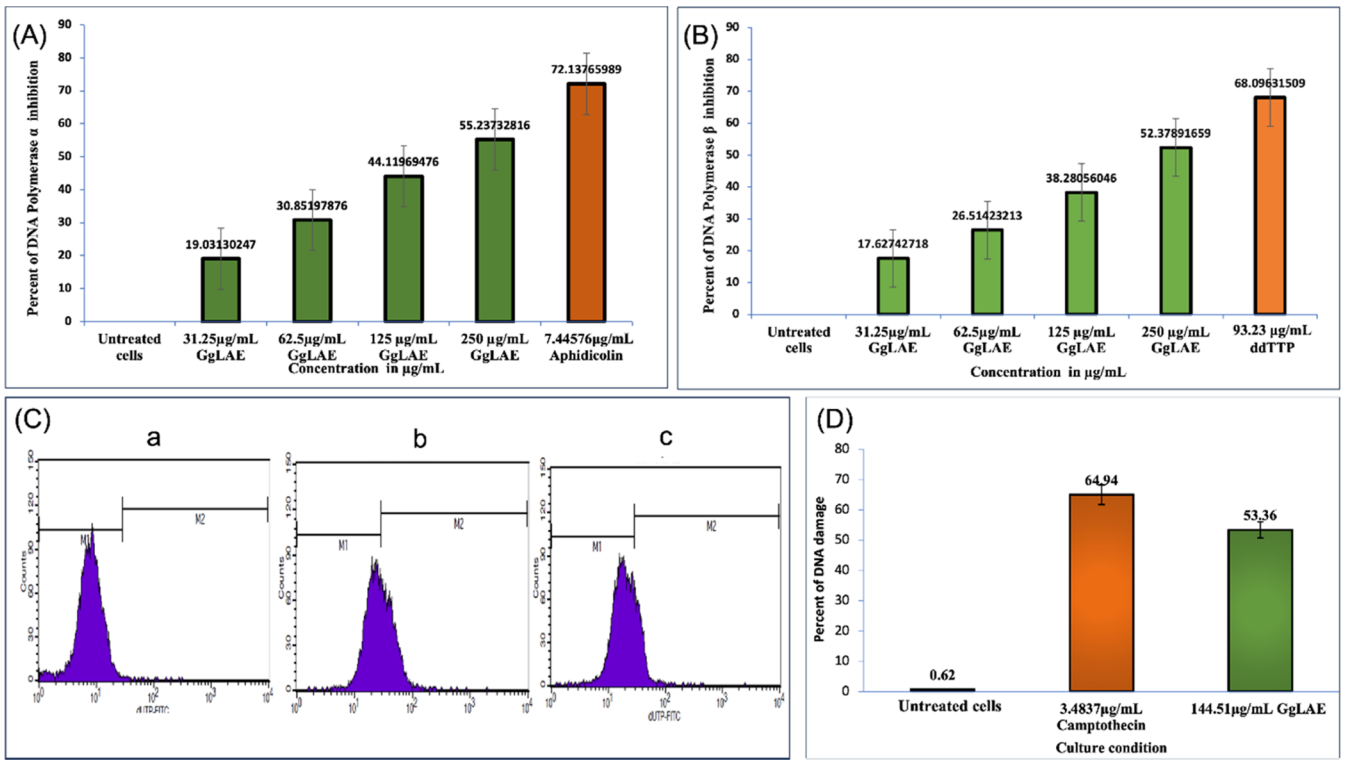

Figure 3. Confirmation of cell death due to apoptosis in MCF-7 cells induced by GgLAE. (A) Inhibition of mammalian DNA polymerase α activity by GgLAE. (B) Inhibition of mammalian DNA polymerase β activity by GgLAE. (C) Confirmation of DNA damage (dUTP expression) by TUNEL assay: (a) untreated cells, (b) camptothecin (3.4837 μg/mL), and (c) GgLAE (144.51 μg/mL). (D) Overlay bar graph depicting the percent of cells expressing dUTP-FITC (expression of dUTP is directly proportional to DNA damage).

that GgLAE contains spindle poisons that have the potential to inhibit tubulin polymerization and arrest cell cycle in MCF-7 cancer cells.

Tubulin polymerization assay is crucial in understanding tubulin dynamics and regulation. It allows the investigation of tubulin assembly and disassembly into microtubules, essential components of the cell's cytoskeleton.[30] Certain plant extracts and alkaloids have been reported to affect tubulin polymerization.[4] Results obtained from the tubulin polymerization assay using GgLAE demonstrated a dose- and time-dependent reduction in tubulin polymerization (Figure 2C). The graph depicted a decrease in absorbance values over time as the concentrations of the alkaloid extract increased from 15.125 to 250 μg/mL, indicating a decline in tubulin polymerization. Lower concentrations of GgLAE (15.125, 31.25, and 62.5 μg/mL) exhibited a gradual decrease in absorbance values, while the higher concentrations (125 and 250 μg/mL) showed a more rapid decrease. Overall, the results of the tubulin polymerization assay suggest that GgLAE possesses alkaloids with the potential to inhibit tubulin polymerization. Similar findings were reported for extracts of *Alstonia penangiana*.[31]

2.6. Mitochondrial Membrane Potential (JC-1) Assay. Mitochondrial membrane potential ($\Delta\psi$) of MCF-7 cells was determined using JC-1 dye on the basis of the red-to-green fluorescence ratio. Results confirmed that GgLAE contains the alkaloids that affect mitochondrial membrane potential (Figure 2D,E). The UR region (Figure 2E) represents the % of cells with high mitochondrial membrane potential (normal/healthy cells with polarized mitochondria), and the LR region (Figure 2E) represents the % of cells with low mitochondrial membrane potential (dead/unhealthy cells with depolarized mitochondria). In the figure (Figure 2E), FL1 on the *X*-axis stands for green fluorescence and FL2 (Figure 2E) on the *Y*-axis stands for red fluorescence. The untreated MCF-7 cells (control) had the highest mitochondrial membrane potential with a mean value of 430.16 for the high $\Delta\psi$ (measured by red fluorescence), compared to camptothecin-treated cells (positive control treated) and cells treated with the GgLAE, which had mean values of 128.89 and 172.49, respectively (Figure 2D,E). The low $\Delta\psi$, measured by green fluorescence was highest for camptothecin-treated cells with a mean value of 49.87, followed by cells treated with the GgLAE (mean value of 36.23), and untreated cells (mean value of 12.63). The red-to-green fluorescence ratio was highest for cells treated with camptothecin (mean value of 2.58), followed by GgLAE (mean value of 4.76), and untreated cells (mean value of 34.06). Overall, the results suggest that the GgLAE affects the red-to-green fluorescence ratio and mitochondrial membrane potential and induces mitochondrial dysfunction, which may have therapeutic applications in cancer treatment.[27]

The JC-1 assay is particularly useful for studying processes related to mitochondrial health, such as mitochondrial dysfunction, apoptosis, and cellular metabolism.[32] Some plant alkaloids have been found to possess various pharmacological properties and can interact with cellular components, including mitochondria.[33]

In the study of JC-1 staining to assess the depolarization of mitochondrial membrane potential (MMP), which is considered an early apoptosis event, treatment of HepG2 cells with *Syzygium cumini* (Myrtaceae) extract caused the fluorescence to change from red to green, showing the loss of MMP. These findings are comparable to the results of the present study in MCF-7 cells where GgLAE affects the red-to-green fluorescence ratio and affects mitochondrial membrane potential.

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2.7. DNA Polymerase α and β Inhibition Assays. The effect of GgLAE on DNA polymerase α activity was investigated by conducting assay at different concentrations tested, and aphidicolin was used as a standard positive control at 7.4457 μg/mL. Results confirm that GgLAE exhibited a dose-dependent decrease in DNA polymerase α activity with higher concentrations of GgLAE leading to greater inhibition of activity[24] (Figure 3A). Standard deviation and standard error calculations were performed for each concentration. These findings suggest that GgLAE exerted an inhibitory effect on DNA polymerase α activity in a dose-dependent manner. Notably, the highest concentration tested (250 μg/mL) demonstrated significant inhibition with a remarkable 55.23% reduction compared to that of the untreated control. These results highlight the presence of DNA polymerase α inhibitory alkaloids in GgLAE, and thus, GgLAE possesses potential therapeutic applications as a DNA polymerase α inhibitor, indicating its promise for the treatment of abnormal DNA replication diseases.[25]

Similarly, the DNA polymerase β assay was conducted with varying concentrations of GgLAE alongside an untreated control and standard ddTTP (93.23 μg/mL). Results revealed a dose-dependent inhibitory effect of GgLAE on DNA polymerase β activity (Figure 3B). At the highest concentration (250 μg/mL), GgLAE displayed significant inhibition, with an impressive 68.10% reduction in DNA polymerase β activity compared with the untreated control. These findings suggest the presence of DNA polymerase β inhibitory alkaloids in GgLAE.

DNA polymerases α and β are enzymes that play crucial roles in DNA replication and repair. Inhibition of these enzymes activities can lead to genome instability and cell death.[34] In the present report, the inhibitory effect of GgLAE on DNA polymerases α and β was investigated using a fluorometric assay. Results showed that GgLAE inhibited the activity of both DNA polymerases in a dose-dependent manner, with increasing inhibition observed at higher concentrations of GgLAE. Compared to the untreated control, GgLAE inhibited the activity of DNA polymerase α by 17.6% at 31.25 μg/mL and by 68.1% at 250 μg/mL. While the inhibition of DNA polymerase β was 26.5% at 31.25 μg/mL and 72.1% at 250 μg/mL (Figure 3A,B). The inhibitory effect of GgLAE on both enzymes suggests that it may have potential as an anticancer agent, as cancer cells rely heavily on DNA replication and repair pathways for their survival.

Collectively, the alkaloids present in GgLAE demonstrated substantial inhibition of DNA polymerase α and β activities, highlighting GgLAE's potential as a promising therapeutic agent for diseases associated with abnormal DNA replication like cancer. However, further research is required to investigate the mechanism of inhibition, and the potential toxicity of GgLAE. In certain studies, researchers have explored the impact of plant alkaloid extracts on DNA replication or repair pathways, indirectly involving DNA polymerases.[35] Similar findings were reported for chloroform extract of *Chonemorpha fragrans* callus that was found to possess potent anticancer potential, exhibiting DNA polymerase inhibitory activity.[36]

2.8. TUNEL Assay for Apoptosis. Results of the TUNEL assay using flow cytometry analysis indicated that in the MCF-7 cell line, the untreated cells had a very low level of DNA damage (0.62% positive cells), while cells treated with camptothecin, a standard drug had a significantly higher level of DNA damage (64.94% positive cells). The cells treated with GgLAE (144.51 μg/mL) showed DNA damage (53.36% positive cells), promoting cell death by apoptosis. These findings suggest that GgLAE can potentially induce DNA damage in MCF-7 cells, albeit lower than camptothecin. These results are promising, as DNA damage is a key mechanism by which cancer cells are killed or prevented from dividing.

The increase in the percentage of cells showing positive FITC-dUTP staining in the M2 region following GgLAE treatment indicates the induction of DNA damage, which is a sure sign of apoptosis (Figure 3C,D). These findings suggest that GgLAE may trigger apoptosis via the DNA damage-induced pathway.

The TUNEL assay was developed by Gorczyca et al., in 1992.[37] The assay identifies the DNA fragmentation in cells undergoing apoptosis (programmed cell death). TUNEL assay utilizes the enzyme terminal deoxynucleotidyl transferase (TdT) to label the free ends of DNA fragments with labeled nucleotides. However, in particular research studies, plant alkaloid extracts have been investigated for their potential to induce or inhibit cell apoptosis.[38] In the present study, results of the TUNEL assay using flow cytometry analysis indicated that in the MCF-7 cell line the untreated cells had a deficient level of DNA damage (0.62% positive cells).

Interestingly, comparing the 3D culture model to the 2D culture showed that apoptosis dramatically increased in the 3D model following treatment with the *Astragalus hamosus* extract. Moreover, depending on the culture model, the extract had distinct effects on cell cycle regulation. In the 3D model, the extract interrupted the S and G2/M phases of the cell cycle, whereas the G0/G1 phase was impacted in the 2D culture.[39] These findings suggest that GgLAE may trigger apoptosis via the DNA damage-induced pathway. However, further studies are required to explore the specific molecular mechanism of GgLAE-induced apoptosis and evaluate its potential as a therapeutic agent against breast cancer.

3. CONCLUSIONS

The outcome of the present study demonstrates the cytotoxicity and anticancer potential of the *G. glauca* (Fresen.) Gilg leaf alkaloid extract to inhibit cellular processes involved in cancer cell growth and division. The extract was confirmed to possess cytotoxic alkaloids that inhibit tubulin polymerization, alter mitochondrial membrane potential, and inhibit DNA polymerase α and β activity results based on a dose- and time-dependent manner. The GgLAE induced cell cycle arrest at G2/M and S phases in MCF-7 cells, inhibiting cell division and proliferation. The extract also induced DNA damage in MCF-7 cells, suggesting its potential to affect cancer cell growth and survival. However, further studies are needed to fully understand the extract's efficacy, potential toxicity, and ability to induce apoptosis in cancer cells. Overall, these findings provide valuable insights into the potential of *G. glauca* (Fresen.) Gilg as a good source of natural phytochemical compounds with therapeutic applications in treating cancer and other diseases involving uncontrolled cell division, mitochondrial dysfunction, and DNA replication.

4. MATERIALS AND METHODOLOGY

4.1. Plant Materials. Fresh and healthy leaves of *G. glauca* (Fresen.) Gilg were collected during the daytime from the Western Ghats in India near Kundadri Hill basin, Shivamogga district, Karnataka, India. The plant's authentication was done

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by Dr. Kumaraswamy Udupa, a renowned plant taxonomist from the Department of Botany, Sri JCBM College, Sringeri, Chikkamagaluru district, Karnataka, India. A voucher specimen (FSB-0982) was preserved in the department's herbarium. Subsequently, the leaves were cleaned, shade-dried, and ground into a coarse powder.

4.2. Extraction. Leaf powder of *G. glauca* was defatted with *n*-hexane before subjecting to a 6 h Soxhlet extraction using 10% acetic acid in ethanol. Extract was filtered and concentrated to one-fourth of its initial volume by recovering the solvent using a rotary evaporator (Roteva, Medica Instruments, India). Thus, the obtained extract was subjected for isolation of leaf alkaloid extract/fraction by washing twice with diluted ammonium hydroxide and filtration. Residue was dried in a hot-air oven at 40 °C to attain a consistent weight, which was anticipated to contain alkaloids and this was confirmed by qualitative analysis.[8,9]

4.3. RP-HPLC Analysis of GgLAE. Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed using an Agilent 1260 infinity HPLC system equipped with a diode array detector (DAD). Alkaloid extract of *G. glauca* leaves has been dissolved in methanol (1 mg/mL). By injecting 20 μL of the *G. glauca* leaf alkaloid extract (GgLAE) and keeping the flow rate at 1 mL/min, separation was carried out via a Zorbax SB-C-18 column (4.6250 mm, 5 m). Separated analytes were picked up at 210 nm in the mobile phase, which was composed of methanol and water at a ratio of 35:65. The exact temperature of the column was kept at 35 °C. The standards used were caffeine and colchicine (1 mg/mL in methanol or ethanol).

4.4. Liquid Chromatography−High-Resolution Mass Spectrometry (LC-HRMS). Alkaloids found in GgLAE were confirmed and identified using LC-HRMS (liquid chromatography with high-resolution mass spectrometry). The injection volume of the sample (GgLAE) 1 mg/mL in ethanol was about 5 μL. Mobile phase was composed of two parts: mobile phase A, which included 0.1% formic acid and 100% water, and mobile phase B, which contained 100% acetonitrile or 100% methanol, and was conducted for 35 min at a pressure of 1200 bar. An Agilent Hypersil GOLD C18 column with dimensions of 100 mm × 2.1 mm and a particle size of 3 m was used for the analysis. Data from the ReSpect (RIKEN MSn spectrum database for phytochemicals), METLIN, and HMDB databases were used to identify the compounds.

4.5. Cell Line and Cell Culture. The MCF-7 human breast cancer cell line was utilized to test GgLAE's cytotoxicity and anticancer properties. MCF-7 cells were defrosted in a water bath heated to 37 °C, pelleted down by centrifugation for 5 min at 1000 rpm, and then rehydrated in DMEM. A hemocytometer was used to count the cells, and the cell density was set at 1−2 105 cells/mL. In a 37 °C humidified 5% CO_2 incubator, cells were sown in a 75 cm^2 culture flask. The culture medium was changed every 2−3 days or when the cells achieved 80% confluency in order to maintain optimum growth. For passage, the culture medium was first removed, and the flask was washed with phosphate-buffered saline (PBS). Next, 2−3 mL of a trypsin-EDTA solution was added to the flask, and the cells were then detached from the flask surface by incubating the flask at 37 °C for 1−2 min. Addition of DMEM with FBS neutralized the trypsin activity. Finally, cells were moved to a fresh culture flask or plate for additional testing.[10]

4.6. Cytotoxicity Assessment by MTT Assay. By employing a colorimetric test and the water-soluble tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Cytotoxicity of *G. glauca* leaf alkaloid extract (GgLAE) was evaluated in MCF-7 (human breast cancer) cells, and MCF-10A cells. Further, *G. glauca* leaf alkaloid extract (GgLAE) purified using a syringe filter PVDF membrane with a 13 mm diameter and 0.45 μm pore size[11] to obtain *G. glauca* leaf alkaloid fraction (partially purified) and was assessed in MCF-7 (human breast cancer) cells, HT-29 (human colon cancer) cells, and HeLa (human cervix cancer) cells (data not shown). By converting yellow formazan (MTT) crystals into insoluble formazan chromophore crystals that are light purple when dissolved in the proper solvent (DMSO), the test measures cell proliferation.[12] Test sample GgLAE was dissolved in DMSO (0.5 mg/mL) and used for cytotoxicity testing at various concentrations (ranging from 12.5 to 200 g/mL). It was then incubated with 200 L of seeded cell suspensions of the MCF-7 cell line in a 96-well microplate overnight at 37 °C with 5% carbon dioxide and the remaining 95% air. Insoluble formazan chromophore purple crystals were shown to develop in the mitochondria of living cells. After the formazan crystals were dissolved in DMSO, optical densities at 570 nm were measured using a microplate reader. To get the IC_{50} values, the linear regression equation $Y = Mx + C$[13] was employed.

4.7. Cell Cycle Analysis Using Propidium Iodide. In this study, MCF-7 cells were cultured in a 6-well plate at a density of 2×10^5 cells/2 mL and incubated overnight at 37 °C for 24 h. Following this, the spent medium was aspirated, and the cells were treated with the GgLAE at its IC_{50} concentration (144 μg/mL), along with appropriate controls in 2 mL of culture medium, followed by an additional 24 h incubation period. For sample preparation, the cells were detached using a trypsin-EDTA solution, harvested into polystyrene tubes, and centrifuged. The cell pellet underwent fixation in 70% ethanol, followed by washing with PBS. To selectively stain DNA, the cell pellet was treated with a propidium iodide (PI)/RNase staining buffer and incubated in the dark. Flow cytometry analysis was performed on the samples in PI/RNase solution without additional washing steps.[14]

4.8. Tubulin Polymerization Assay. The following chemicals were used to create the tubulin polymerization buffer: 80 mM PIPES (pH 6.8), 2 mM $MgCl_2$, and 0.5 mM EGTA. 1 mM GTP was added to the buffer and carefully mixed. To get rid of any aggregates, the tubulin solution was defrosted on ice and centrifuged at 10,000*g* for 10 min. The polymerization buffer was then used to dilute tubulin to a final concentration of 10 M. 10 μL of tubulin solution was put into each well of a 96-well microplate. 10 μL of DMSO was added to one row of wells as a negative control, and 10 μL of nocodazole (3.0126 g/mL) was added to another row as a positive control. The remaining microplate wells were exposed to concentrations of 15.125, 31.25, 62.5, 125, and 250 μg/mL in 10 μL of GgLAE (0.5 mg/mL) in DMSO. Each well's contents were gently blended using pipetting. The microplate was sealed with a lid and incubated at 37 °C for 30 min to avoid evaporation. A 96-well microplate reader was used to measure each well's absorbance at 340 nm after incubation. Data were plotted, and tubulin polymerization in several wells was contrasted.[15]

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4.9. Mitochondrial Membrane Potential (JC-1) Assay. The Invitrogen MitoProbe JC-1 Test Kit was used to perform the mitochondrial membrane potential test. 100 μL was used to distribute the MCF-7 cell suspension (5 × 104 cells) into each well of a 96-well plate. Each well received 100 μL of the GgLAE solution (IC_{50} value: 144.51 μg/mL) in order to reach the appropriate final concentration. DMEM-high glucose served as the untreated control group (negative control), whereas camptothecin (3.4837 μg/mL) was administered to the positive control group. The plate was then placed in a humid incubator and incubated for 24 h at 37 °C with 5% CO_2. Following the manufacturer's instructions, JC-1 (5,5,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimi-dazoylcarbocyanine iodide) dye was created. The medium from each well was carefully evacuated following the 24-h compound treatment, and 100 μL of JC-1 dye (1×) was then applied to each well. For 15 min, the plate was heated to 37 °C in the dark. After aspirating the JC-1 dye, 200 μL of D-PBS was used to wash the wells. Each well received 100 μL of brand-new D-PBS before the cells were examined using a flow cytometer. Each treatment group's proportion of polarized (red) and depolarized (green) cells was noted.

Each well's JC-1 dye was aspirated for microscopic visualization before being washed with 200 μL of D-PBS. The cells were then examined using a 200X inverted biological microscope (Biolink) and 100 μL of fresh D-PBS added to each well. The images of the cells were captured on film. The proportion of depolarized (green) and polarized (red) cells in each treatment group was calculated using flow cytometry data. To assess the impact of the test substance (GgLAE) on the mitochondrial membrane potential ($\Delta\Psi$m) of MCF-7 cells, the results of the GgLAE-treated cells were compared to the untreated cells (negative control).[16]

4.10. DNA Polymerase α and β Inhibition Assay. DNA polymerase α and β inhibitor assay mix was made by dilution of 10× reaction buffer (stock) to 10-fold with nuclease-free water. We made a 100-fold dilution of each stock in nuclease-free water to create a 2 mM dNTP solution and a 10 μM template primer DNA solution. With the use of nuclease-free water, DNA polymerase α and β enzymes were also diluted to a final concentration of 1 unit/μL. Different concentrations of the GgLAE test sample were created by dilution of the stock (0.5 mg/mL) to final concentrations of 31.25, 62.5, 125, and 250 μg/mL using nuclease-free water.[17] Aphidicolin (7.445576 μg/mL) and ddTTP (200 μM) were used as positive controls for DNA polymerase α and β, respectively. Assay reaction assembly: The assay mix was created by mixing the following ingredients in a 1.5 mL microcentrifuge tube: 5 μL of 10× reaction buffer, 2 μL of 2 mM dNTP solution, 1 μL of 10 mM template primer DNA solution, 1 μL of DNA polymerase or enzyme (1 unit/μL), and the proper quantity of either the test sample (GgLAE) or positive control (aphidicolin 7.445576 μg/mL for DNA polymerase α or ddTTP- 200 μM for DNA polymerase β). For appropriate mixing, tubes were quickly vortexed, centrifuged, and then incubated at 37 °C for 60 min. After that, they were stopped by heating at 95 °C for 10 min. By counting the amount of PicoGreen dye fluorescence present in the reaction mixture, the efficiency of DNA synthesis and polymerization was determined. The reaction mixture was transferred to a 96-well. Each well received 100 μL of PicoGreen dye, and the plate was kept in the dark and at room temperature for 10 min. Using a BioTek Synergy HT Microplate Reader (Agilent Technologies) with an excitation at 485 nm and emission at 535 nm, fluorescence was detected. The results of the calculation of the relative fluorescence units (RFUs) were graphed. To evaluate the impact of GgLAE on the DNA polymerases α and β, the fluorescence of the test sample was compared to the positive control and untreated sample (negative control).[18] To determine the percentage of activity inhibition of DNA polymerase(s), the following equation was used

$$\%\ \text{inhibition} = \frac{(\text{mean RFU of untreated control} - \text{mean RFU of test samples})}{\text{mean RFU of untreated control}} \times 100$$

4.11. TUNEL Assay for Apoptosis. By using the TUNEL test, apoptotic DNA fragmentation was found. The test substance (GgLAE) and the positive control stock solutions were prepared by dissolving camptothecin in DMSO at a dosage of 10 mg/mL. To attain the appropriate final concentrations, the cell culture medium was used to dilute stock solutions, yielding IC_{50} values of 144.51 μg/mL for the GgLAE test sample and 3.4835 μg/mL for camptothecin, with DMSO serving as the negative control. 1.5×10^5 MCF-7 cells per well in 2 mL of complete media were planted in a 6-well cell culture plate that was incubated at 37 °C overnight. Carefully removing the culture medium, D-PBS was used to wash the cells. Each well received 2 mL of completely new media containing the test chemicals, and cells were cultured for 24 h at 37 °C. Cells were carefully separated from their medium, twice-washed in D-PBS, and then fixed in 1 mL of 70% (v/v) ethanol per well at 4 °C for at least an hour. After carefully removing the ethanol, the cells had two rinses with reaction buffer containing the TdT enzyme and FITC-dUTP, and 50 μL was added to each well. The reaction solution was then kept at 37 °C for 1 h in a humid incubator. After carefully removing the reaction solution, the cells were washed twice with wash buffer, 50 μL of PI/RNase staining buffer was added to each well, and the reaction solution was then incubated for a further 30 min at room temperature. Using a flow cytometer with a 488 nm excitation filter and a 530 nm emission filter, cells were examined.[19,20] To evaluate the proportion of positive cells and the mean fluorescence intensity (MFI), BD CellQuest Pro ver.6.0 software was used. To ascertain the degree of apoptosis produced by the test compounds, the results of GgLAE-treated cells were compared with those of cells treated with the negative and positive control compounds.[21]

4.12. Statistical Analysis. Data of each investigation/experiment were collected from three independent trials, and statistical analysis was conducted using MS Excel and represented as mean ± SE. One-way ANOVA was employed for data analysis in MS Excel. The significance of *G. glauca* extracts was determined based on a statistical difference of $p < 0.05$.

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Notes

The authors declare no competing financial interest.

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