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Goniothalamus lanceolatus extract inhibits the growth of human ovarian cancer cells through migration suppression and apoptosis induction

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ABSTRACT

Several studies have revealed the medicinal potential of *Goniothalamus lanceolatus* (GL) for various diseases. This study aims to examine the effects of GL extracts on cell viability, migration, and apoptotic rate of chemosensitive and chemoresistant ovarian cancer cells. The dissolved GL leaf and root extracts in dichloromethane (DCM), hexane, and methanol were tested against PEO1 (chemosensitive) and PEO4 (chemoresistant) cell lines. MTT (3-(4,5-Dimethylthiazol2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to determine the cell viability after 24 hours exposure to various extract concentrations at two-fold serial dilutions (0–1,000 µg/ml). A scratch assay was used to examine cell migration. The Fluorescein isothiocyanate (FITC) flow cytometry was used to observe cell apoptosis. The results showed that GL leaf and root extracts inhibit the viability of PEO1 and PEO4 cells in a dose-dependent manner. The lowest 50% inhibitory concentration values for PEO1 were 31.40 ± 0.77 µg/ml in methanol root extracts while they were 22.02 ± 0.52 µg/ml for PEO4 in DCM leaf extracts. Cell migration rate was suppressed at 18.65% in PEO1 and 16.73% in PEO4 after 72 hours treatment compared to the untreated cells. The extract caused high percentages of early apoptosis in PEO4 (26.5%) compared to PEO1 (2.67%) after 48 hours treatment. Significant inhibitory effects of GL extracts on cell viability, cell migration, and apoptosis in both cell lines suggest its potential as a chemotherapeutics candidate for ovarian cancer. However, further downstream studies are needed to evaluate the cellular mechanisms.

INTRODUCTION

Ovarian cancer is known to be one of the most aggressive gynecological cancers causing death in women. An estimated 240,000 new cases have been recorded in 2018, leaving ovarian cancer at ranking number seven as the most common cancer among women worldwide (Henderson *et al*., 2018). Ovarian cancer cases have been mostly identified in the advanced stages due to their asymptomatic characteristics (Jayson *et al*., 2014). Nonetheless, some patients may experience bloating, pelvic pain, frequent urination, and changes in bowel movements (Jayson *et al*., 2014). Cisplatin is a standard drug used for ovarian cancer treatment; however, the cell resistance has been shown to cause relapse in some patients following initial treatment (Noviyani *et al*., 2019). This substantially reduced the effectiveness and outcome of chemotherapy and resulted in less than 30% for a 5-year survival rate (Cornelison *et al*., 2017). Hence, it is extremely crucial to discover alternative approaches to treat ovarian cancer, especially in overcoming cisplatin resistance with minimal side effects on patients.

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Goniothalamus is one of the largest paleotropical genera of a plant in the Annonaceae family, comprising over 130 species and is mainly distributed in the Malesian floristic region, comprising Malaysia, Borneo, New Guinea, Sumatra, and Philippines (Saunders, 2003; Yang *et al*., 2020). Goniothalamin (GTN) as the main bioactive compounds has been reported to exert a cytotoxic effect and anticancer properties in human promyelocytic leukemia (HL-60), leukemia monocytic (U937) (Petsophonsakul *et al*., 2013), squamous cell carcinoma (H400 cells) (Li *et al*., 2016), and cervical cancer (HeLa) cell lines (Sophonnithiprasert *et al*., 2017). The compound is capable of limiting the development of cancer cells by increasing the number of dead cancer cells without producing inflammatory reactions on the healthy cells (Seyed *et al*., 2014; Umar-Tsafe *et al*., 2004).

Goniothalamus lanceolatus (GL) is native to the rainforest in Sarawak, Malaysia, locally known as "Getimang," and is believed by the Iban people to repel mosquitoes due to the pungent scent and thick smoke when burned. It has also been used to treat cold, fever, and skin diseases by the indigenous people as alternative medicine (Wiart, 2007). The isolation of the pure compounds and the cytotoxicity chemical classification of the active compounds are currently ongoing. The newly discovered alkaloids of goniolanceolactam and 2-acetyl-3-amino-1,4-naphthoquinone isolated from the dichloromethane (DCM) root extract were reported to have a cytotoxic effect on human colon and lung cancer cells (Rasol *et al*., 2018a). Moreover, eight new bis-styryllactones and goniolanceolatins A−H, and four known styryllactones with a rare (6S)-styrylpyrone and (1S)-pyranopyrone moieties were also isolated from the GL (Bihud *et al*., 2019). These compounds have been reported to be associated with antimalarial properties against chloroquine-sensitive (3D7) and chloroquine-resistant (K1) strains of *P. falciparum* (Kaharudin *et al*., 2020). Apart from these findings, antiproliferative and anticancer activities of GL on ovarian cancer cells have not yet been reported. Therefore, this study aims to investigate the cell viability, migration, and apoptotic effects of GL extracts in three different solvents on chemosensitive and chemoresistant ovarian cancer cells.

MATERIALS AND METHODS

Preparation of plant extracts

GL leaves and roots were collected from Sematan Sarawak, Malaysia, in June 2012, described as FBAUMS 108 by a botanist Professor Dr. Kamaruddin Mat Salleh (Universiti Kebangsaan Malaysia). The leaves and the roots of the plants were oven-dried before being subjected to extraction using DCM, hexane, and methanol solvents. The solvents were removed under a vacuum condition using a rotary evaporator resulting in a total of six extracts. The extracts were purified using high-performance liquid chromatography (HPLC) and recycling HPLC leaving the isolation of a series of compounds (Rasol *et al*., 2018b). The extracts were reconstituted in dimethyl sulfoxide (DMSO) to form a 20 mg/ml stock solution and preserved for further use at –20°C.

Cell culture

Two ovarian cancer cell lines, namely, PEO1 (chemosensitive) and PEO4 (chemoresistant), were provided by Dr. Normala Abd. Latip of the Atta-ur-Rahman Institute for Natural Product Discovery, which was previously purchased from the European Cell Culture Collection (ECACC, UK). The ovarian cancer cell lines were derived from ascites of the same patient who received cisplatin, 5-fluorouracil, and chlorambucil treatment. PEO1 was sensitive to cisplatin taken when the patient was able to respond to the treatments. Meanwhile, PEO4 was taken 10 months later after the patients developed resistance toward cisplatin chemotherapy. RPMI-1640 complete media containing 10% fetal bovine serum and 1% penicillin-streptomycin were used for cell culture and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Seeding of monolayer cell cultures was performed in a 75 cm^2 tissue culture flask and maintained up to 70% confluency prior to treatment.

MTT assay

Cell viability and inhibition were determined using the MTT assay. Briefly, each cell line was plated in a 96-well plate seeded with 2.0×10^4 cells/well followed by 24 hours cell treatment with different extract concentrations at two-fold serial dilutions (0–1,000 µg/ml). Cell viability was determined by adding 5 mg/ml MTT solution to each well. After 4 hours of incubation, 50 µl of DMSO was added to each well as a stop solution and incubated for 10 minutes at room temperature on a shaker. The absorbance was measured at 570 nm wavelength using a microplate reader (BMG Labtech, Offenburg, Germany). A graph of cell viability percentage versus extract concentration was plotted and the inhibitory concentration (IC_{50}) was determined from the concentration-response inhibition curves. Cells without treatment (untreated cells) were used as negative control (NC) while cells treated with cisplatin were used as a positive control. The extracts with the lowest IC_{50} value were selected for subsequent testing.

Scratch assay

The migration rate of both cell lines was determined using a scratch assay. Briefly, the cells were cultured in a six-well plate until it reached 70% confluency. The cells were then treated with the extracts and cisplatin (positive control). A scratch was introduced using a sterile pipette tip followed by 16, 24, 48, and 72 hours incubation period. The closure of the scratched wounds was considered as the completion of the migration process. The migration rate of both cells was determined by comparing the migration rate of the treated cells with the untreated cells (negative control). The following formula was used to calculate the migration rate (Warden *et al*., 2020):

$$\text{Rate of migration (\%)} = \frac{\text{Area of scratch at 0 hour} - \text{area of scratch at } (n) \text{ hour}}{\text{Area of scratch at 0 hour}} \times 100$$

where n = time of incubation.

Cell apoptosis assay by flow cytometry

Cell apoptosis was assessed by the BD Pharmigen™ FITC Annexin V (AV) Apoptosis Detection Kit as per the manufacturer's protocol. A cell density of 2×10^6 cells/well was seeded in a six-well plate, followed by cell treatment and incubation for 24 and 48 hours. The cells were harvested by

trypsinization, washed three times with cold phosphate-buffered saline (PBS), pelleted, and resuspend pellet in a 1× binding buffer. The suspension was then stained with 5 µl FITC-conjugated AV and 5 µl propidium iodide (PI) and incubated for 15 minutes at room temperature in a dark space, followed by the addition of 400 µl binding buffer. Finally, the samples were analyzed by a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA). The cell distribution was analyzed using CellQuest™ software (Becton-Dickinson) within 1 hour of staining in which 10,000 cells were collected for each sample. Samples with different cell groups were separated in either early or late apoptosis, marked by AV while necrosis was marked by PI and presented in a separate plot group.

Statistical analysis

The statistical analysis test was carried out using GraphPad Prism Software version 6.0. Data are presented as mean ± standard mean error (SEM). The statistical comparative analysis was performed using one-way analysis of variance, followed by Tukey's *post-hoc* test. A *p*-value of less than 0.05 was considered to be significant.

RESULTS

Effect of GL extracts on cell viability and cell inhibition

Figure 1 shows the cell viability in PEO1 after 24 hours of treatment with different concentrations of the extracts. DCM and methanol leaves extract significantly reduced the

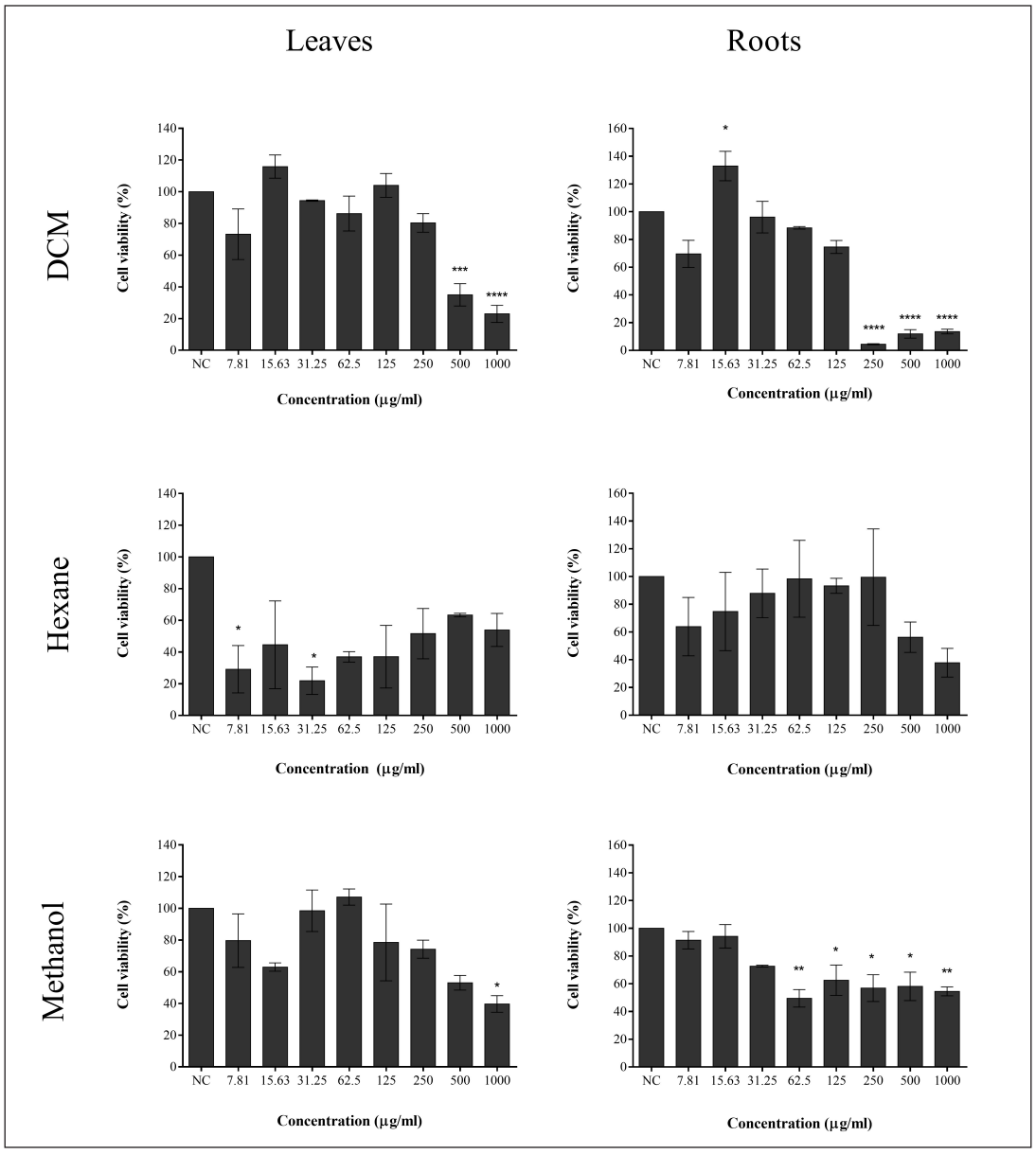

Figure 1. Cell viability of the PEO1 cell line treated with GL leaf and root extracts. Each leaf and root extract was dissolved in DCM, hexane, and methanol and treated at different concentrations for 24 hours in triplicate. All data are expressed as mean ± SEM. **p* < 0.05; ***p* < 0.01,; *****p* < 0.0001 versus negative control (NC).

cell viability ($p < 0.05$) at the highest concentration (1,000 µg/ml) after 24 hours of treatment. A significantly decreased cell viability to below 20% was observed in DCM roots extract at 250 µg/ml and above concentration while methanol extract decreased the cell viability at around 50% at a concentration of 62.5–1,000 µg/ml.

Figure 2 shows the cell viability in PEO4 after 24 hours of treatment with different concentrations of leaf and root extracts of GL. The viability of the cells decreased significantly by the DCM leaves extract at a concentration of 1,000 µg/ml compared to the hexane leaves extract at 500 µg/ml. In addition, the DCM root extracts significantly decreased the cell viability ($p < 0.0001$) to less than 20% at 125–1,000 µg/ml concentration. The methanol root extract showed a significant reduction of less than 50% cell viability with a concentration of 7.81–1,000 µg/ml compared to the control group.

Figure 3 shows the cell viability in both cell lines after 24 hours of cisplatin treatment. In the PEO1 cell line, the viability decreased significantly at 1.56 µM and almost 90% inhibition was observed at 6.25 µM as shown in Figure 3a. A nonsignificant cell viability decrease was seen in PEO4 after cisplatin treatment except at 3.125 µM as shown in Figure 3b.

Table 1 summarizes the IC_{50} of the leaf and root extracts in both cell lines. The lowest IC_{50} values for PEO1 were methanolic root extracts (IC_{50}: 31.40 ± 0.77 µg/ml). DCM leaf extract (IC_{50}: 22.02 ± 0.52 µg/ml) showed a moderate IC_{50} value in PEO4. Cisplatin inhibited PEO4 almost twice the concentration as seen in PEO1 (IC_{50}: 4.07 ± 0.21 µM *vs*. 2.76 ± 0.05 µM).

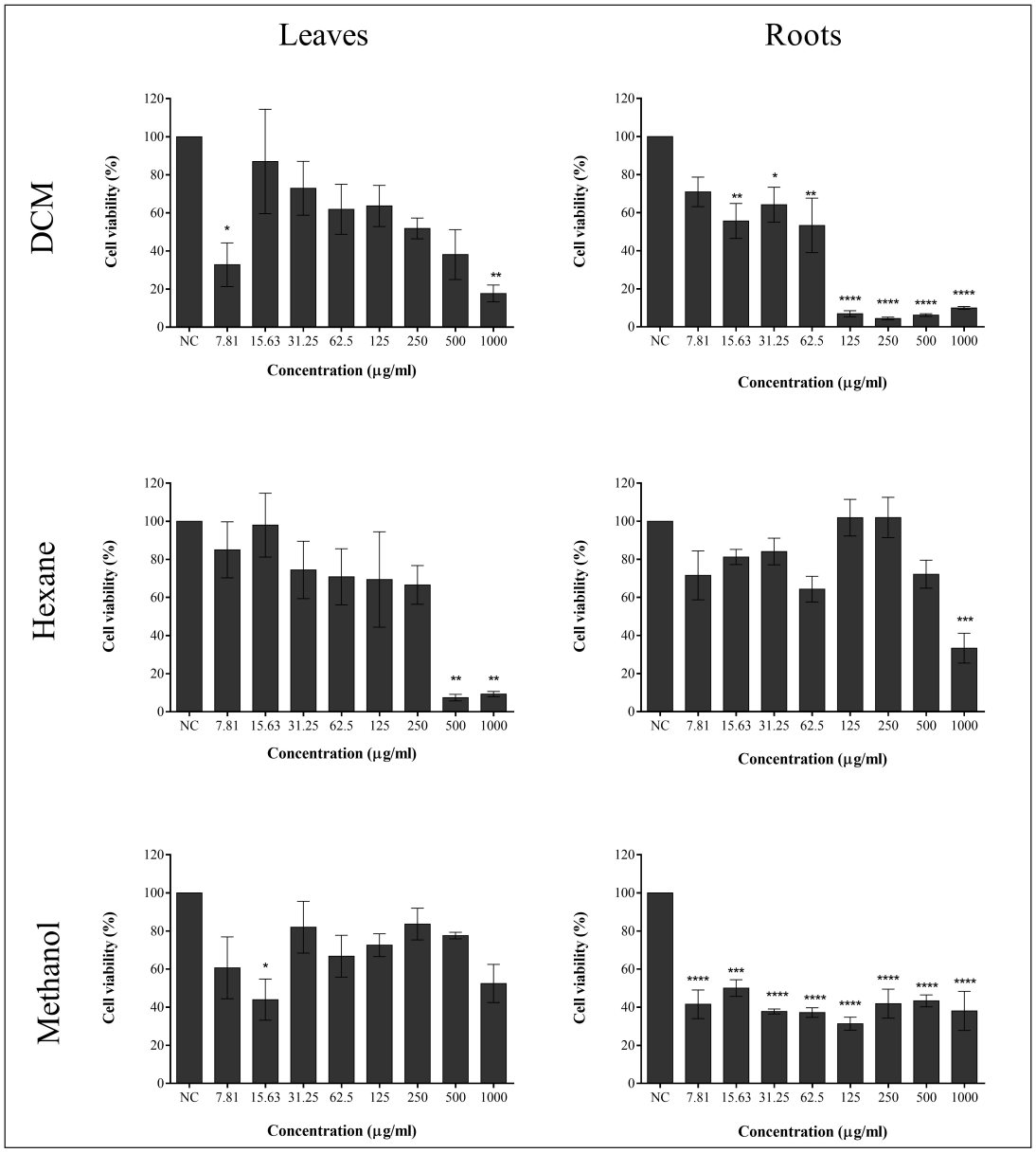

Figure 2. Cell viability of the PEO4 cell line treated with GL leaf and root extracts at different concentrations for 24 hours in triplicate. All data are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$,; ****$p < 0.0001$ versus negative control (NC).

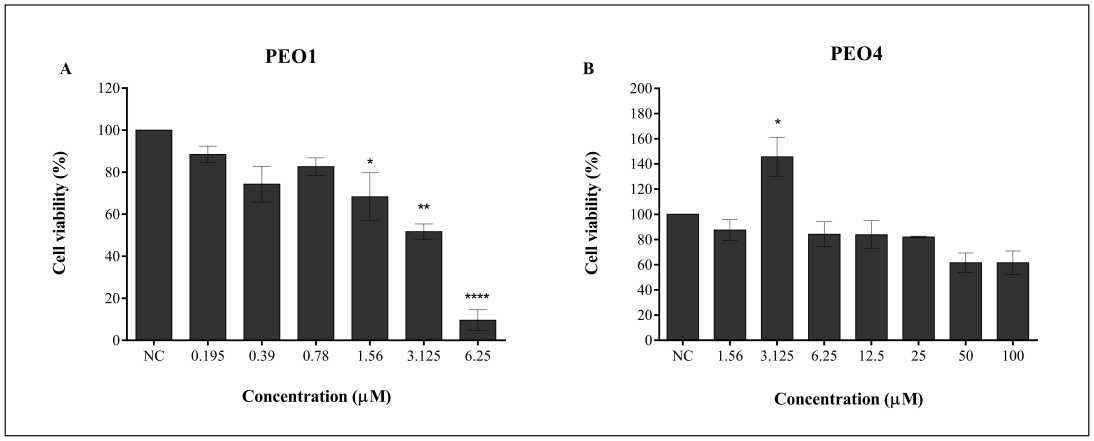

Figure 3. Effect of cisplatin on cell viability of (A) PEO1 and (B) PEO4 cell lines. Cisplatin was used as a standard drug for ovarian cancer cell lines and served as a positive control. Both cell lines were treated with cisplatin in increasing concentrations in triplicate for 24 hours and determined by MTT assay. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ versus negative control (NC).

Table 1. The IC_{50} values of GL leaf and root extracts for PEO1 and PEO4 cell lines.

Plant part	Extraction solvent	IC_{50} ± SEM (µg/ml)	
		PEO1	PEO4
Leaves	DCM	166.10 ± 0.15	22.02 ± 0.52
	Hexane	170.80 ± 0.12	66.46 ± 0.16
	Methanol	114.50 ± 0.35	54.19 ± 0.29
Root	DCM	80.24 ± 0.14	78.65 ± 0.03
	Hexane	71.75 ± 2.90	472.60 ± 0.39
	Methanol	31.40 ± 0.77	72.51 ± 0.26
Cisplatin	Positive control	2.77 ± 0.05 µM	4.07 ± 0.21 µM

*The extract with the lowest IC_{50} in each cell line was used for further testing.

Effect of GL on cell migration

The extract with the lowest IC_{50} value from the cell viability analysis for each cell line was used to assess cell migration. PEO1 cells were treated with methanolic roots extract at $IC_{20,}$ IC_{50}, and IC_{80} concentrations at 16, 24, 48, and 72 hours time points, respectively (Fig. 4). At 72 hours, cell migration decreased from 21.37% at IC_{20} to 18.65% at $IC_{80.}$ In comparison, untreated cells showed a 76.63% cell migration. Cisplatin was able to fully inhibit PEO1 cell migration (–2.49%).

PEO4 cells were treated with DCM leaf extracts at $IC_{20,}$ IC_{50}, and IC_{80} concentrations and incubated at four different time points (Fig. 5). At 72 hours, cell migration was suppressed to 16.73% at IC_{80} compared to 31.99% in the untreated cells. Meanwhile, the migration was almost the same in the untreated cells when treated with cisplatin (30.3%).

Effects of GL in inducing apoptosis

The effects of the extracts on inducing apoptosis using flow cytometry were examined at 24 and 48 hours. In PEO1, cells treated with methanol root extracts at IC_{20} and IC_{80} showed increased early apoptosis to 6.76% and 13.52%, respectively, after 24 hours. The cells at IC_{20} and IC_{80} shifted to late apoptosis at 4.48% and 6.64%, respectively. Early apoptosis significantly decreased while the cells shift toward late apoptosis after 48 hours of treatment as shown in Figure 6A–C.

In PEO4 cells, DCM leaves extract significantly increased early apoptosis at IC_{20} to 42.94% but dropped after 24 hours to 24.63% at IC_{80}. However, early apoptosis at IC_{20} and IC_{80} increased to 16.28% and 26.5%, respectively, after 48 hours. Late apoptosis increased significantly at both dose-dependent times as shown in Figure 7A–C.

DISCUSSION

Cell viability assay following the administration of GL methanol root extract gave the lowest IC_{50} value at 31.40 ± 0.77 µg/ml in PEO1. However, a higher concentration at 72.51 ± 0.27 µg/ml was needed to inhibit PEO4. The results showed that methanol root extract exhibited a higher cytotoxic effect against chemosensitive cells compared to the chemoresistant cells. However, DCM leaves extract was found to be the most cytotoxic to the chemoresistant cells, PEO4 (IC_{50}: 22.02 ± 0.52 µg/ml) compared to the chemosensitive cells and PEO1 (IC_{50}: 166.10 ± 0.15 µg/ml). The selection of chemosensitive and chemoresistant cells was done to compare the effects of the plant extracts on cells toward different chemosensitivity profiles.

Chemoresistance in the ovarian cancer cells was initiated by the cell adaptation using different energy pathways such as glycolysis or oxidative phosphorylation (Dar *et al*., 2017). Chemosensitive ovarian cancer cell lines (A2780 and PEO1) appeared to exhibit a glycolytic phenotype and could not tolerate

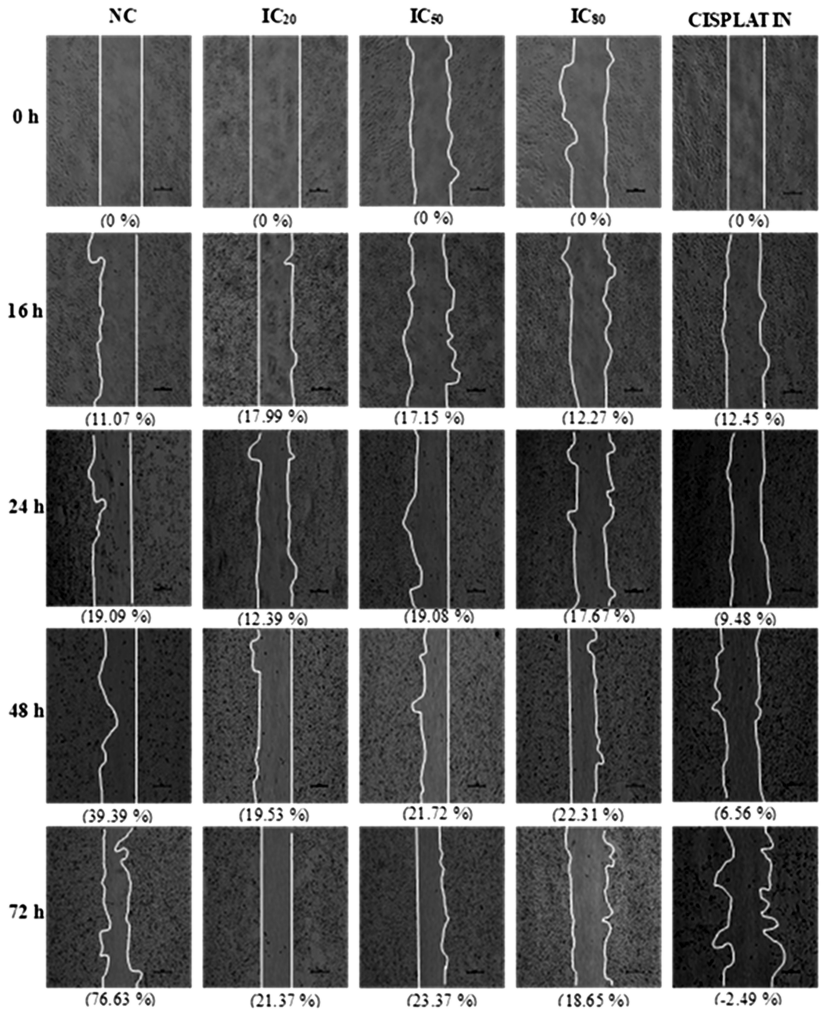

Figure 4. Effect of GL methanol root extract on PEO1 ovarian cancer cell migration by scratch assay. The experiments were repeated thrice to determine the average percentages of cell migration.

glucose deprivation. However, the chemoresistant counterparts (C200 and PEO4) showed a high metabolically active phenotype and the ability to switch between oxidative phosphorylation and glycolysis (Dar *et al*., 2017). Cell proliferation can be suppressed by apoptosis through inhibition of signal transducers and transcription activator 3 and activation of Janus kinase 2 (Jo *et al*., 2012). Alonezi *et al*. (2016) demonstrated that chemoresistant cells (A2780CR) were highly sensitive to melittin and had a slightly lower IC_{50} value of 4.5 µg/ml compared to 6.8 µg/ml in the chemosensitive cells (A2780).

The GL leaves and roots were extracted using different solvents (DCM, hexane, and methanol) of different polarities to obtain different groups of active compounds. DCM is a moderate polar organochloride solvent that is widely used to dissolve most organic compounds. Hexane is a nonpolar solvent commonly used in the extraction of nonpolar compounds and methanol is a polar and universal solvent. Due to these differences, the presence and quantity of the compounds will also affect the activity of the cells. The phytochemical analysis indicates the presents of goniodiol, 8-epi-9-deoxygoniopypyrone 9-deoxygoniopypyrone, digoniodiol, and GTN in the GL extract (Zohdi *et al*., 2017). Two new alkaloids, goniolanceolactam and 2-acetyl-3-amino-1,4-naphthoquinone, were isolated from the DCM root extract of the GL (Rasol *et al*., 2018a). Goniolanceolactam showed cytotoxic activity on human colon and lung cancer cell lines with IC_{50} values ranging from 5.32 to 9.91 µM. A new styryl lactone, 5R,6R-5-hydroxy-6-styryltetrahydropyrane-2-one, was isolated from the GL roots with cytotoxic activity of IC_{50}: 2.38–7.59 µM against human colon and lung cancer cell lines (Rasol *et al*., 2018b).

PEO1 and PEO4 cells are primarily derived from patients with poorly differentiated serous adenocarcinoma at different stages of ovarian cancer. The cells were found as single adhesive cells or small clusters *in vitro* with approximately 37 hours of doubling times (Langdon and Lawrie, 2001). Cancer cell migration is generally associated with the alteration of the cell-matrix interface on the cell surface (Kim *et al*., 2012). Inhibition of cell migration prevents cancer metastasis and enhances patient survival *in vivo* (Helbig *et al*., 2003). Therefore, it was hypothesized that GL extracts could modulate cell migration while controlling disease progression. The scratch assay revealed

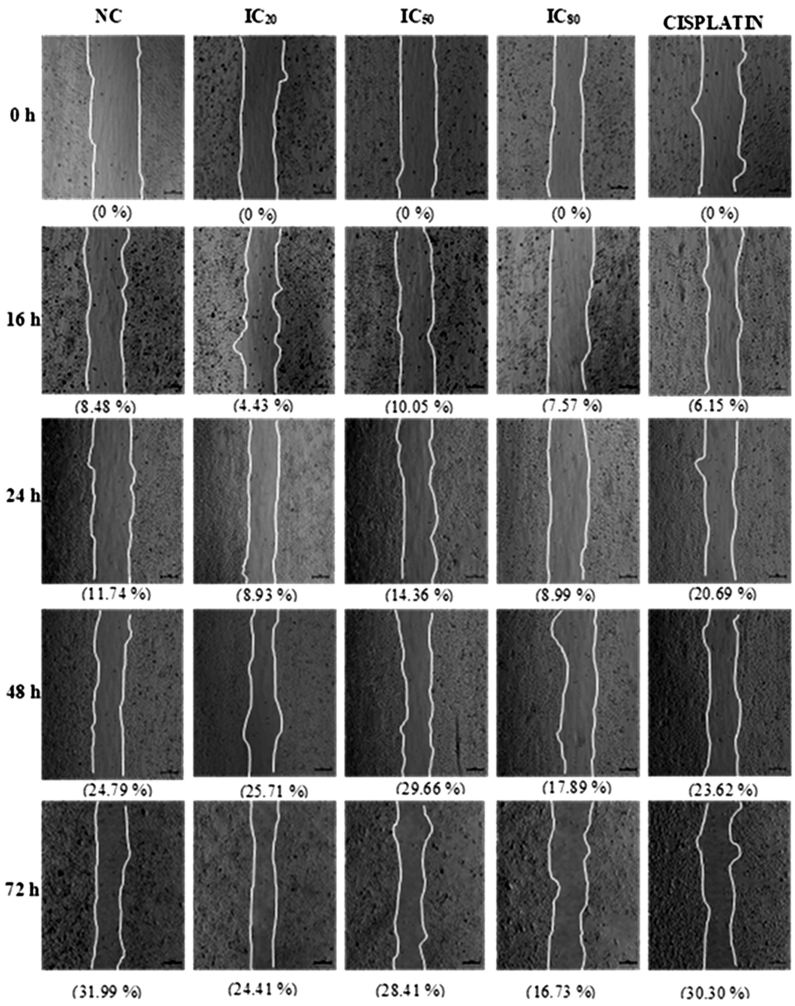

Figure 5. Effect of GL DCM leaves extract on PEO4 ovarian cancer cell migration assessed by scratch assay. The experiments were repeated thrice to determine the average percentages of cell migration.

that the GL extracts inhibited cell migration in a concentration-dependent manner compared to the negative control. Cells treated with a higher concentration of GL extracts inhibited cell migration more than with a lower concentration. Interestingly, suppression of cell migration by the GL extract was found to be dependent on the type of cell resistance, whereby it inhibited more chemoresistant cells than chemosensitive cells compared to the negative control cells.

A previous study showed that GTN inhibits human lung cancer cell line migration (H1299) at a concentration of less than 10 µg/ml. The inhibition of migration is associated with decreased levels of metalloproteinase matrix (MMP-2 and MMP-9) activity (Chiu *et al*., 2011). Another study found that Goniolactone-C from *G. cheliensis* strongly inhibited PDGF-BB-induced vascular smooth muscle cell (VSMC) migration by the suppression of adhesion molecule expression (Sun *et al*., 2014). Similarly, auraptene and *Kaempferia parviflora* extract suppressed cell migration and invasion of ovarian cancer *in vitro* by inhibiting the MMP-2 and MMP-9 activities (Jamialahmadi *et al*., 2018; Paramee *et al*., 2018).

Apoptosis is essential in a multicellular organism and is a dominant tumor-suppressive pathway, which can potentially deplete cancer cells (Ghante and Jamkhande, 2019). Cancer cells have the ability to a range of modifications and responses. Mutation is one of the modifications that can cause dysfunction to the apoptotic machinery pathway, thus rendering the cancer cells to be resistant to the drug (Das *et al*., 2016). Apoptosis has been shown by the externalization of phosphatidylserine indicating early apoptosis in cell death with intact membrane integrity. Necrotic cells are the late apoptotic cells with damaged membranes (Foo *et al*., 2014). This study showed that GL extracts induced apoptosis in both cell lines; however, GL has a higher ability to induce apoptosis in chemoresistant cells compared to the chemosensitive cells.

This finding was similar to the previous studies that showed GTN from *G. griffithii* increased early apoptosis in MDA-MB-231 breast cancer cells and an increased level of caspases 3, 8, and 9 activities (Khaw-On *et al*., 2018). In addition, GTN from *G.macrophyllus* also induced early apoptosis and triggered S-phase arrest of HeLa cells after 24, 48, and 72 hours of treatment

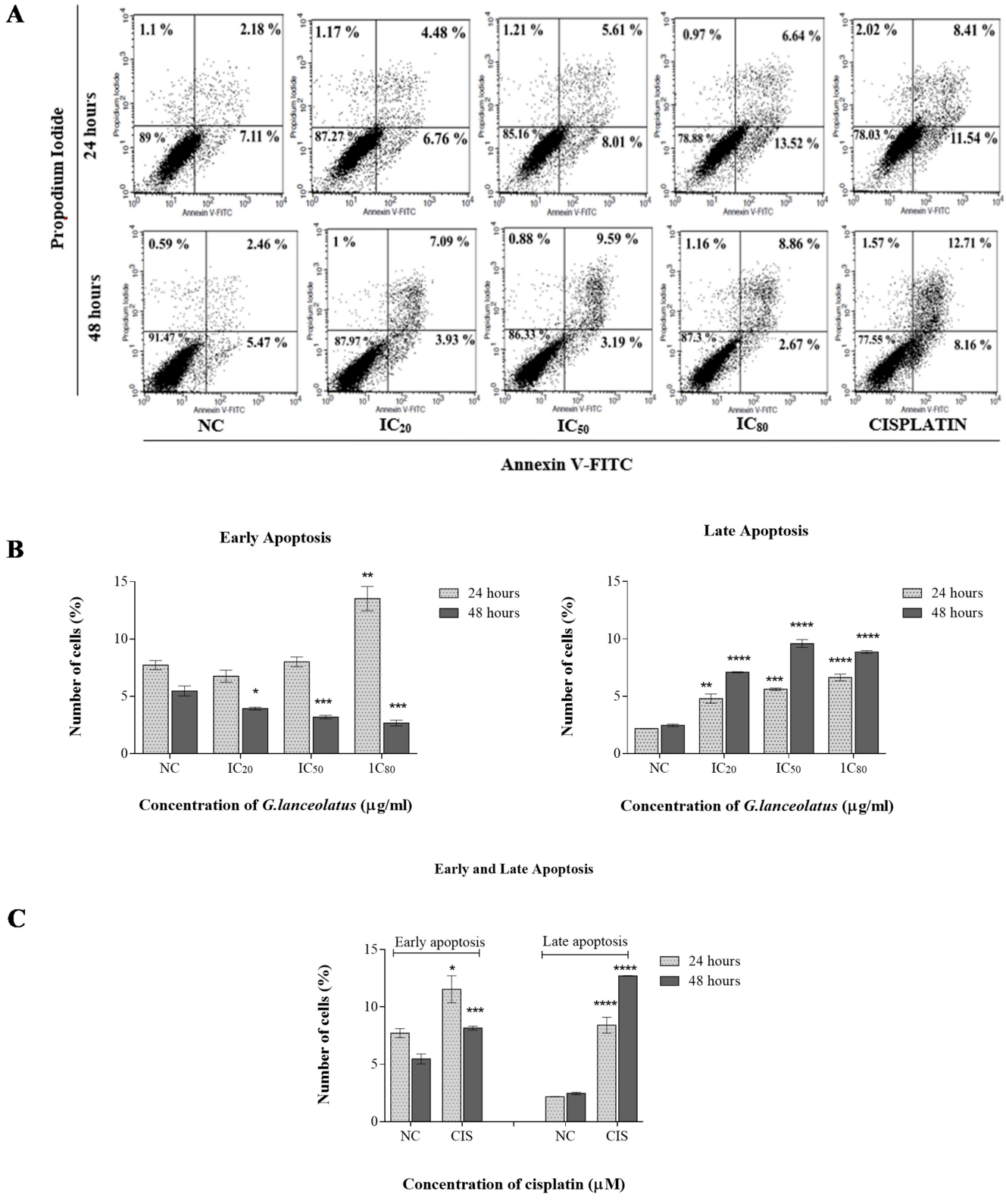

Figure 6. Effect of GL methanol root extract on PEO1 cell apoptosis. (A) Cell populations are shown in quadrants: the proportion of viable cells, necrotic cells, early apoptotic cells, and late apoptotic cells is shown in the lower left, upper left, lower right, and upper right, respectively. (B) and (C) Percentages of early and late apoptosis shown in the bar graph. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ versus negative control (NC).

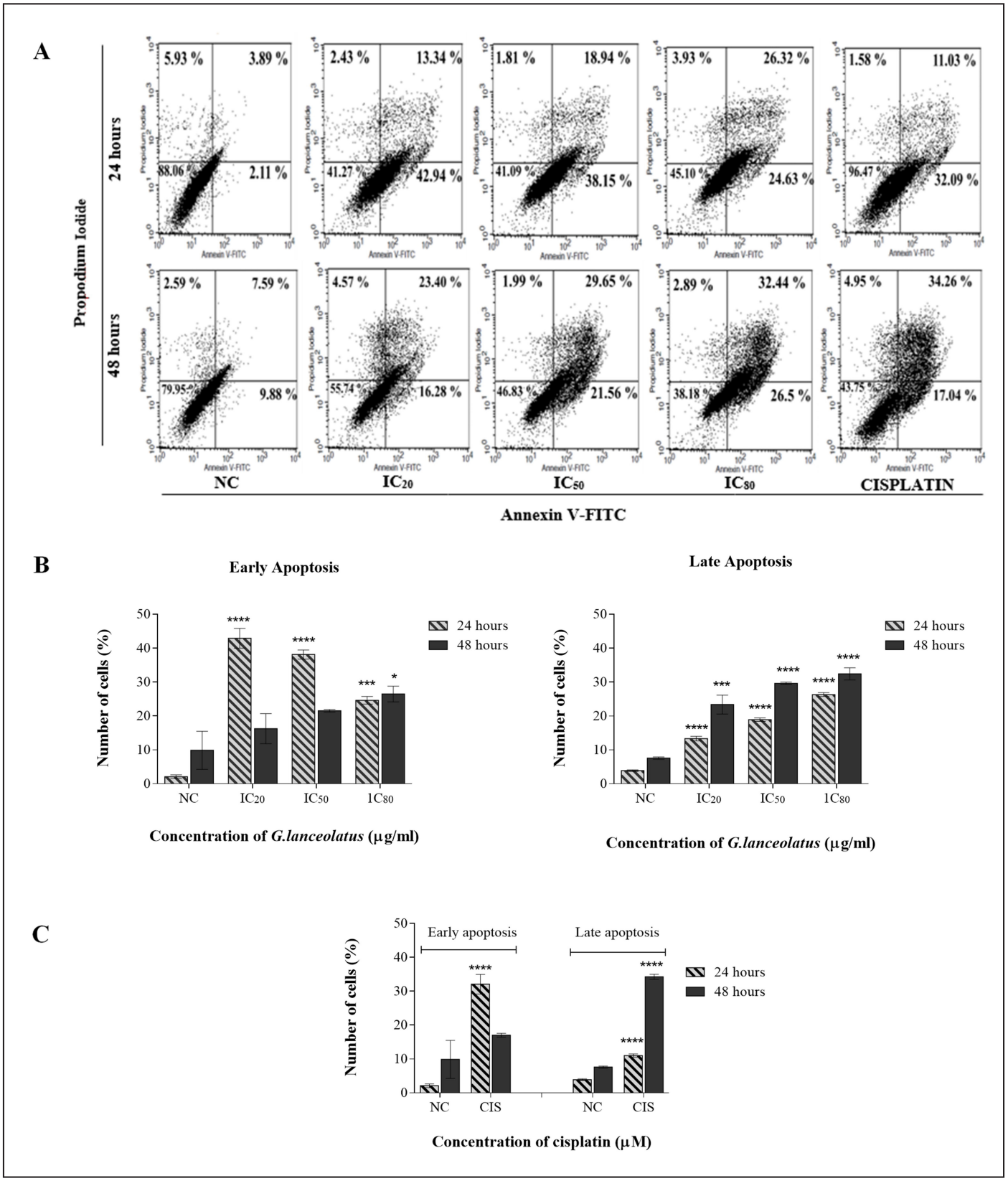

Figure 7. Effect of GL DCM leaves extract on PEO4 cell apoptosis. (A) Cell populations are shown in quadrants; the proportion of viable cells, necrotic cells, early apoptotic cells, and late apoptotic cells is shown in the lower left, upper left, lower right, and upper right, respectively. (B) and (C) Percentages of early and late apoptosis shown in the bar graph. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ versus negative control (NC).

(Alabsi *et al*., 2012). It also induced apoptosis in MCF-7, HeLa, HepG2, and NIH3T3 cell lines, with IC_{50} values of 7.33, 14.8, 37.1, and 65.4 µM, respectively (Banjerdpongchai *et al*., 2016). Tangchirakhaphan *et al*. (2018) in another study revealed that GTN mediates chromatin condensation and apoptotic bodies in A375 skin cancer cells.

CONCLUSION

Goniothalamus lanceolatus extracts exhibited a significant inhibitory effect on cell viability, cell migration, and apoptosis initiation in ovarian cancer cells (PEO1 and PEO4). Nonetheless, careful attention is needed to better understand these promising findings and their interaction as natural extract products containing a complex mixture of natural compounds and may involve multiple molecular targets and pathways.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This type of study does not require ethical approval.

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GRAPHICAL ABSTRACT