RESEARCH ARTICLE

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In vitro Evaluation of Anti-Cancer Potential of Different Solvent Extracts Derived from Clerodendrum Infortunatum Linn against Cervical Cancer

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Abstract

Background: Cervical cancer is a prevalent and deadly malignancy in females, with chemotherapy often proving ineffective due to significant side effects and the development of chemo-resistance. This study investigates the medicinal potential of *Clerodendrum infortunatum linn.*, a genus with approximately 500 species in the Lamiaceae family. Limited research exists on the species of *Clerodendrum infortunatum* and its various solvent extracts. **Objective:** The study aims to assess the anti-cancer properties of different solvent extracts from this plant on human cervical cancer cells. **Methods:** The study examines the plant's phytochemical components and their potential to inhibit cancer growth. Aerial parts of the plant were extracted using the Soxhlet method, and the presence of Rutin, Quercetin, and Gallic Acid in specific solvent extracts was validated through High-Performance Thin Layer Chromatography (HPTLC). In vitro assays, including MTT, Apoptosis, Cell Cycle analysis, Intracellular Reactive Oxygen Species assessment, and Gene expression PCR, were conducted to investigate the plant's anti-cancer properties further. **Results:** The outcomes of the phytochemical assessment indicated that Rutin was predominantly present in the water extract, with quercetin being more concentrated in the decoction, and the hydro-alcoholic extract showing elevated levels of gallic acid. Notably, the decoction extract demonstrated the highest cytotoxic activity, primarily through early apoptosis and arrests in the S-phase and G2M phases. *Clerodendrum infortunatum* exhibited a reduction in Intracellular Reactive Oxygen Species. The gene expression analysis disclosed an impact on the *BCL-2* gene. **Conclusion:** Notably, *Clerodendrum infortunatum* exhibited the ability to initiate early apoptosis, halt the cell cycle at the S and G2M phases, and diminish levels of reactive oxygen species significantly. The gene expression analysis revealed an influence on the *BCL-2* gene. To sum up, this research underscores the encouraging cytotoxic and antioxidant attributes of Clerodendrum infortunatum, implying its potential for cervical cancer treatment.

Keywords: *Clerodendrum infortunatum*- HeLa cell line- Apoptosis- Anti-oxidant activity- gene expression

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Introduction

According to the findings of Sathishkumar et al., it was determined that the projected count of newly diagnosed cancer cases in India for the year 2022 amounted to 1,461,427, with a crude rate of 100.4 cases per 100,000 individuals. Alarmingly, the statistics indicate that one out of every nine individuals in India is at risk of developing cancer at some point in their life. Notably, lung cancer stood as the most prevalent form of cancer among males, while breast cancer ranked as the primary type of cancer among females. The insights derived from these estimations can play a pivotal role in devising targeted initiatives aimed at cancer prevention and control. These efforts may encompass early detection interventions, strategies for reducing risk factors, and effective management approaches [1].

Extensive research efforts are dedicated to the development of more efficient treatments with reduced side effects. The focus lies in innovating therapies that can specifically target and eliminate malignant tumor cells while leaving normal cells unharmed. This pursuit aligns with the ultimate objective of achieving cancer chemotherapy where selective drugs can either eradicate cancerous cells or induce their transition into non-malignant states [2]. Addressing this challenge

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practically involves turning to traditional herbs as potential sources for drug development. Traditional medicine systems have heavily relied on plants, playing a pivotal role in their evolution.

Numerous phytochemical compounds and their derived metabolites exhibit a diverse range of pharmacological activities within the human system. This includes but is not limited to flavonoids, phenolics, alkaloids, tannins, gums, oils, glycosides, and resins. These bioactive constituents contribute to the multifaceted therapeutic effects observed in plant-derived medicines [1, 3]. Herbal medications offer a distinct advantage in terms of accessibility to consumers, as they are not only found in drug stores but are also readily available in many other shops. A significant portion of the global population, estimated at around 80%, mainly Asian as well as African nations relies on herbal medicine [2] [4, 5]. Many drugs pivotal to modern medical science have their origins in plants [6].

C. infortunatum, commonly known as the hill glory bower, holds a significant place in Indian folk medicine as a valuable resource. Various species within the Clerodendrum genus have been employed in traditional practices for centuries, showcasing proven antioxidant and hepatoprotective properties. [7, 8]. *C. infortunatum* is widely distributed across the plains of India. Chemical analyses have identified the presence of triterpenes, steroids, and flavonoids in this plant [9, 10]. The plant's documented antioxidant capabilities, [11] antimicrobial effects, [12] anthelmintic properties, [13] and analgesic attributes [14] have sparked increased interest and research endeavors focused on its potential. This growing body of evidence highlights the multi-faceted therapeutic potential of *C. infortunatum* and its prominence in various investigations. The significant role of flavonoids in cancer prevention has been well established, showcasing their considerable impact on both cancer chemoprevention and chemotherapy [3, 15]. A range of flavonoids, including flavanones, daidzein, genistein, quercetin, and luteolin, have demonstrated their efficacy against various human cancers. For instance, they have exhibited effectiveness against breast cancer, [16, 17] and lung cancer [18, 19]. Additionally, quercetin, luteolin, catechin, epicatechin, myricetin, kaempferol, genistein, apigenin, and silymarin have shown significant effects against human prostate cancer [4, 20, 21].

Given this background, the main purpose of the present study was to quantitatively assess the presence of Rutin, Quercetin, and Gallic Acid in *C. infortunatum* and subsequently evaluate the potential anticancer effects of different extracts against a cervical cancer cell line.

Materials and Methods

Plant material

The aerial parts, specifically the leaves, of *C. infortunatum* were sourced from the Vidarbha region in Maharashtra, India. The authenticity and correct identification of the plant materials were established through verification by the Botany Department at the Agharkar Research Institute, (Pune, Maharashtra, India) with the identification code AUTH20-138.

Preparation of extracts

The leaves of *C. infortunatum* were carefully dried for three weeks in the shade, followed by sorting and grinding into coarse powder using a mechanical grinder. Each 50-gram batch of this powder underwent successive extraction through a Soxhlet apparatus, resulting in three distinct types of extracts: Water, Hydroalcoholic, and Decoction. For the Water extract, 50 grams of coarse powder (with a mesh size of 40) were extracted using distilled water at 50 degrees Celsius in a Soxhlet apparatus. The resulting extract was filtered through Whatman filter paper and dried or vaporized in a 45-degree Celsius water bath before being sealed in an airtight container. The Hydroalcoholic extract involved subjecting 50 grams of coarse powder (with a mesh size of 40) to Soxhlet extraction with a mixture of ethanol and distilled water (70:30) at 450C. After filtration through Whatman filter paper, it was dried or vaporized in a 45°C water bath and sealed in an airtight container. For the Decoction, 50 grams of coarse leaf powder were boiled with 800 milliliters of distilled water until the volume reduced to one-fourth. The resulting mixture was filtered using Whatman filter paper at room temperature, and the filtered decoction was vaporized and dried in a water bath. The final product was sealed in an airtight container. The particle size of the final products derived from extraction with water, hydroalcoholic, and decoction samples was determined using the Sympatec GmbH particle assessment system.

Phytochemical analysis

High-Performance Thin Layer Chromatography (HPTLC) was conducted using established protocols to identify and quantify gallic acid, quercetin, and rutin within different solvent extracts of *Clerodendrum infortunatum* [22]. The mobile phase consisted of toluene, ethyl acetate, formic acid, and methanol in a ratio of 3:6:1.6:0.4. The biomarkers used were obtained from LOBA CHEMIE PVT. LTD. To prepare the standard solutions of rutin, quercetin, and gallic acid, separate stock solutions with a concentration of 100 µg/mL were created. Each solution was prepared by taking 10 mg of rutin, quercetin, and gallic acid, individually, and dissolving them in 75 mL of methanol within separate 100 mL volumetric flasks. These mixtures were then subjected to sonication for a duration of 10 minutes, following which the volume was adjusted to the flask's mark using methanol. The resulting standard solutions were then properly filtered using Whatman filter paper in preparation for further analysis.

Cell line and culture conditions

The cancer cell line HeLa (human cervix carcinoma) was acquired from the National Centre for Cell Science (NCCS) Pune, India. These cells were cultured and maintained in Minimum Essential Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L glutamine, and 1% antibiotics (containing 10,000 units of Penicillin, 10 mg of Streptomycin, and 25 µg of Amphotericin B per milliliter). The cells were cultivated at a temperature of 37°C within a humidified atmosphere containing 5% CO_2. Upon achieving a confluence level of 80-90%, the cells were utilized in various assays.

Cytotoxicity Activity

The sub-lethal concentrations (IC_{50}) of extracts and proliferative activity of cells in presence of extracts were determined using MTT assay. Briefly, cultured cells were trypsinised using Trypsin, counted and approximately 2 x 10^4 cells/well was seeded in a 96 well tissue culture plate for cancer cell lines followed by incubation at 37°C, 5% CO_2. After 24 hours, the cells were treated with different concentrations (800, 700, 600, 500, 400, 300, 200 µg/mL) of plant extracts and (25, 20, 15, 10, 5, 1 µg/mL) Doxorubicin (DOXO) for 24 hrs. Thereafter 20 µl of (5 mg/ml) MTT solution was added to each well and incubated for 4 hours at 37°C in 5% CO_2 atmosphere. To terminate the reaction, 100µl of DiMethyl Sulphoxide (DMSO) was added and the plates were gently shaken to dissolve formazan crystals. The intensity of the color developed was measured using an ELISA plate reader at 570nm and a reference wave length of 630nm. All the experiments were performed in triplicates.

Wound scratch assay

An anti-migration assay was conducted using HeLa cells cultured with Doxorubicin and *C. infortunatum*. Initially, 2 x 10^5 cells per ml were seeded into 12-well plates until they achieved a confluence exceeding 90%. Using a 200 µl plastic tip, a linear scratch was created across the center of each well. Subsequently, the scratched cell monolayers were rinsed three times with 1X PBS to eliminate cell debris. The cells were then subjected to a 24-hour incubation, both with and without DOXO and *C. infortunatum*. During this incubation period, photographs of the scratch were captured at 0, 6, and 24 hours, employing an OLYMPUS CKX53 microscope. The cells were allowed to migrate within a medium maintained at 37°C, containing 5% serum, either in the absence or presence of the drugs. This experimental procedure was repeated three times, each time in triplicate, to ensure the consistency and validity of the results [23].

Apoptosis using Annexin V

The FITC Annexin V/Dead Cell Apoptosis Kit from Invitrogen-Molecular Probes® was used for the apoptosis assay. After a 24-hour treatment period, HeLa cells were cold-washed with 1X PBS. Untreated cells served as negative controls, while Doxorubicin-treated cells acted as positive controls. The assay strictly followed the manufacturer's protocol. Flow cytometry analyzed stained cells, focusing on emission wavelengths of 530 nm and >575 nm, enabling assessment of apoptosis in treated cells relative to controls [5, 24].

Cell cycle analysis

After rinsing with 1X PBS and trypsinization, HeLa cells that were both treated with DOXO and *C. Infortunatum* and those left untreated were collected following a 24-hour period. Subsequently, these harvested cells were treated with 25 µl of RNase A (20 mg/ml from Invitrogen), 2 mM $MgCl_2$ (from Sigma), and 5-10 µl of propidium iodide at a concentration of 100 µg/ml (from Invitrogen). The cells were then incubated for an additional 10-15 minutes at room temperature before being subjected to analysis using FACS Caliber from BD Bioscience. This process facilitated the examination of the cells' characteristics using flow cytometry to understand their cellular composition and state [25].

Estimation of intracellular ROS generation

Flow cytometry was used to examine ROS production using DCFHDA [26]. HeLa cells were grown in 6-well plates for 24 hours for this assay. Cells were treated with *C. infortunatum* and DOXO at concentrations of IC_{50} and IC_{25} values and 10 1M DCFH-DA. Untreated cells were incubated with 10 lM DCFH-DA and the maximum amount of DMSO used. Flow cytometry (Beckman Coulter Cytomics FC 500 (kex = 495 nm and kem = 520 nm) was used to measure the generation of fluorescence due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH).

RNA isolation and RT-PCR

Cells were cultured at 5 × 10^4 density in 24-well plates and grown for 24 hours prior to treatment. The cells were then incubated for 4 to 24 hours in a fresh medium with fetal bovine serum and antimycotic-antibiotic. During this time, they were exposed to varying drug concentrations. After the drug incubation, cells were harvested, and total RNA was extracted using TRIzol™ Reagent (Invitrogen, cat no. 15596018), following the manufacturer's instructions. For downstream analysis, isolated RNA underwent reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat no. 4368814) as per the kit's manual. The resulting cDNA samples were stored at -20°C for future use.

The primers used in the present present were as follows:

Gene NAME	SEQUENCE (5'->3')
BaX - F	GGTTGTCGCCCTTTTCTA
BaX - R	CGGAGGAAGTCCAATGTC
BCl-2- R	GAAGACCCTGAAGGACAGCCAT
BCl-2-F	TTGCTTTACGTGGCCTGTTTC
Beta Actin- F	AGAGCTACGAGCTGCCTGAC
Beta Actin- R	AGCATTTCTTCCCGGCCTTT

The comparative CT value analysis followed the established protocol recommended by for Quant Studio 5 Real Time PCR Machine. The protocol involved an initial stage of holding at 50 degrees Celsius for 2 minutes, followed by denaturation at 95 degrees Celsius for 5 minutes. The subsequent PCR stage consisted of 45 cycles involving denaturation at 95° C for 15 seconds, annealing at 54 degrees Celsius, and extension at 72° C for 30 seconds using VeriFlex.

Statistical analysis

The experiments were conducted in triplicate, and the outcomes are reported as the mean value along with the standard deviation (SD). Data analysis was done using GraphPad Prism 8, utilizing ANOVA analysis followed by suitable Post-hoc test. Statistical significance was

determined with a threshold of $p \leq 0.05$.

Results

Phytochemical analysis

The confirmation of the presence of certain pre-determined phytoconstituents, namely Rutin, Quercetin, and Gallic acid in the sample extracts was achieved by comparing the retention factors (Rf values) of the bands obtained in the sample densitograms with those of standard solutions (Table 1). In the densitograms of the standard solutions, distinct peaks were observed at Rf values of 0.74, 0.85, and 0.18 for Rutin, Quercetin, and Gallic acid, respectively (Figure 1). Analyzing the sample densitograms, peaks were detected at Rf values that closely approximated (within Rf ± 0.2) the respective standards under the specific chromatographic conditions employed. Calibration curves were established for Rutin, Quercetin, and Gallic acid, and they demonstrated linearity within the concentration range of 100-2,000 ng/band, respectively. Using the regression equations derived from these calibration curves, the concentrations of Gallic acid, Rutin, and Quercetin present in extracts collected at various time intervals were determined.

The phytochemical analysis revealed distinct concentration patterns among the different types of extracts. In particular, the water extract exhibited the highest concentration of Rutin, surpassing the levels detected in both the hydroalcoholic and decoction extracts. On the other hand, the decoction extract showcased a higher quantity of Quercetin. Notably, the hydroalcoholic extract displayed an elevated concentration of Gallic acid (Table 2, 3; Figure 2).

Cytotoxic activities of Clerodendrum infortunatum on HeLa cell line

The IC_{50} values were ascertained through an assessment of cell viability rates, and the outcomes are outlined in Table 4. The IC_{50} value for the hydroalcoholic extract was 569.04 ± 2.46 µg/ml, the decoction extract exhibited 242.28 ± 3.30 µg/ml, and the water extract recorded 630.03 ± 3.21 µg/ml. Notably, this cytotoxic impact displayed a temporal dependence across all three extract types. Interestingly, the decoction extract demonstrated cytotoxicity at lower concentrations, compared with other extracts, indicating its superior efficacy compared to the other two extracts (Figure 3).

Clerodendron infortunatum alters cell morphology of HeLa cell line

Upon subjecting HeLa cells to treatment with *C. infortunatum* at the IC_{50} concentration for a duration of 24 hours, noticeable alterations in cell morphology were observed. Specifically, following the *C. Infortunatum* treatment, the HeLa cells exhibited an aberrant structural transformation characterized by a rounded and irregular appearance. Remarkably, the capacity of the HeLa cell line to adhere was compromised subsequent to

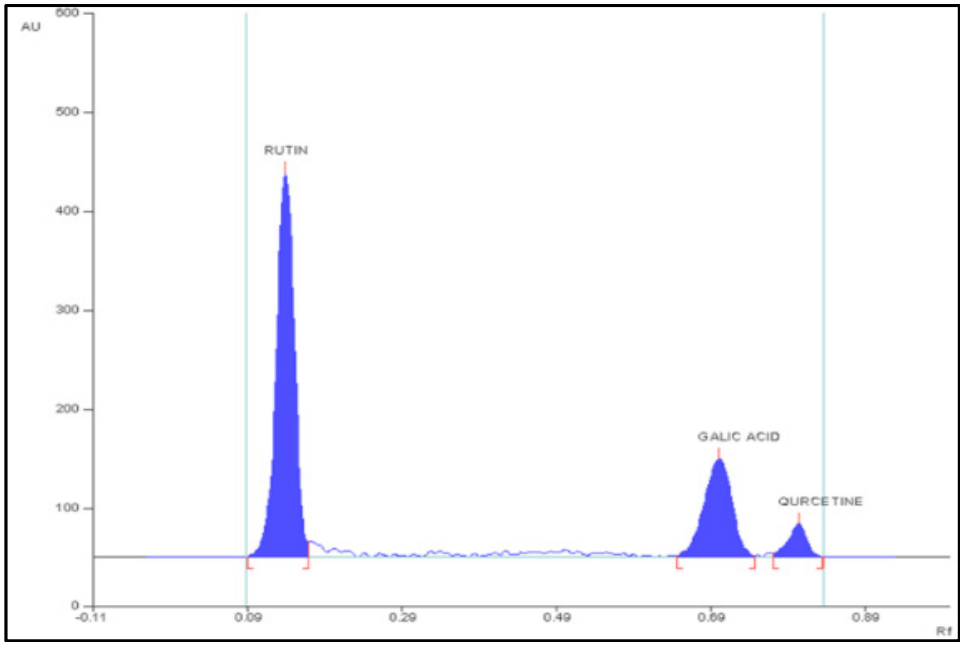

Figure 1. The Densitograms of Standard Rutin, Quercetin, and Gallic Acid

Table 1. Densitograms Values of Standards of Gallic Acid, Quercetin and Rutin

Peak	Start Position	Start Height	Max Position	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.09 Rf	0.4 AU	0.14 Rf	74.46%	0.17 Rf	15.4 AU	7543.3 AU	67.45%	RUTIN
2	0.65 Rf	1.3 AU	0.70 Rf	19.06%	0.75 Rf	0.9 AU	2976 AU	26.61%	GALIC ACID
3	0.77 Rf	3.5 AU	0.80 Rf	6.48%	0.83 Rf	0.0 AU	664.0 AU	5.94%	QURCETINE

Table 2. Estimation of amount of Rutin, Quercetin and Gallic Acid in Different Types of Samples

	Aqueous extract			Hydroalcoholic extract			Decoction		
	Rf	AUC	Conc.(ng/band)	Rf	AUC	conc	Rf	AUC	Conc
Rutin	0.13	1970	8.67	0.12	683	1.81	0.13	1527	4.05
Gallic acid	0.73	1168	7.85	0.72	1200	8.06	0.71	622	4.18
Quercetin	0.81	1249	37.62	0.8	680	20.48	0.78	2347	70.69

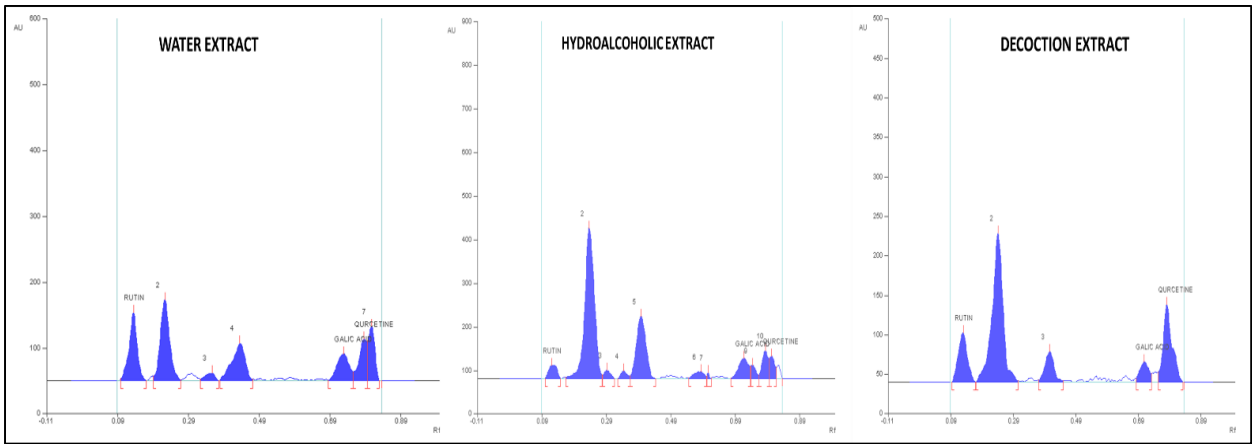

Figure 2. Densitograms of *C. infortunatum* by Soxhlet Method Showing Presence of Rutin, Gallic Acid and Quercetin in Water, Hydroalcoholic and Decoction Extracts Samples.

the *C. infortunatum* treatment (Figure 4).

Effects of C. infortunatum on the migration potential of HeLa cells

At both the 6-hour and 24-hour time points, all three types of extracts displayed a reduction in scratch closure. Similar outcomes were observed when treating with DOXO at its IC_{50} concentration. In the control group, where cells were left untreated by any drugs, the scratched area commenced closure at 6 hours and slight closure

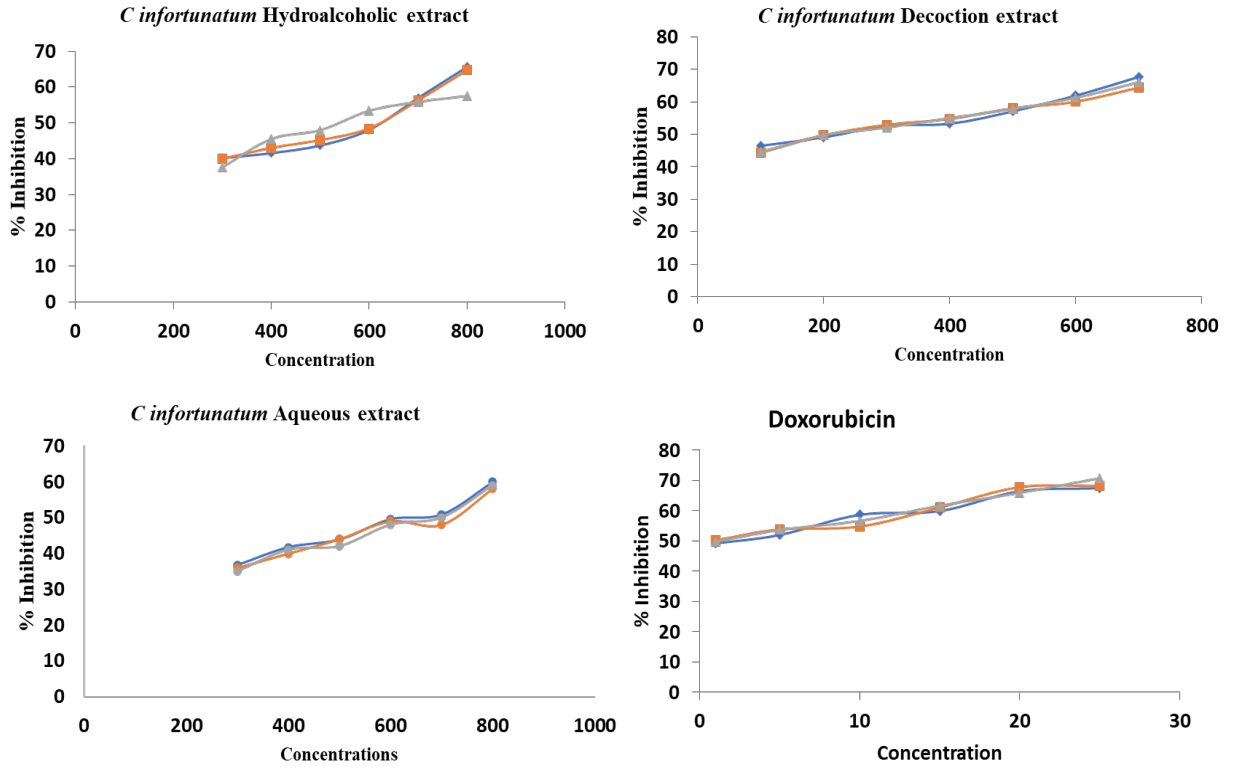

Figure 3. Percent inhibition of *Clerodendrum infortunatum* linn and Doxorubicin on HeLa Cell Line at Different concentrations

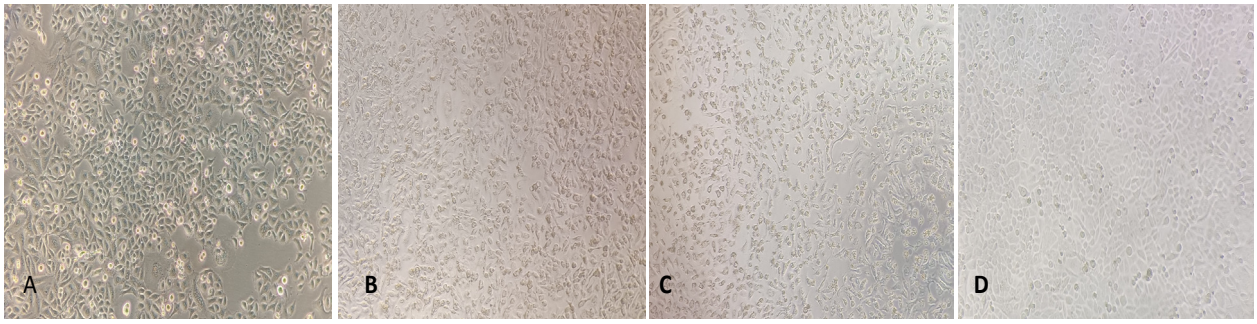

Figure 4. Morphological Changes Observed after *C. infortunatum* linn. treatment on HeLa cells: A) Control: HeLa cells have a normal polygonal shape. B-D) Extract treated cells showed round to slender shape and floating cells that range from irregular to round (Magnification at 200X). (B) Hydro alcoholic (C) Decoction (D) Aqueous

Table 3. Comparison of amount of Rutin, Gallic Acid and Quercetin in All Three Types of Samples of *C. infortunatum*

	AUC		
	Aqueous extract	Hydroalcoholic extract	Decoction
Rutin	1970	683	1527
Gallic acid	68	1200	622
Quercetin	1249	680	2347

by 12 hours. Consequently, it can be inferred that at the IC_{50} concentration, *C. infortunatum* exhibits potential anti-migratory activity (Figure 5).

Evaluation of activity of C. infortunatum on the process of apoptosis on HeLa cells

Numerous signaling and effector pathways, intricately linked to processes such as mitochondrial depolarization, caspase-mediated apoptotic enzyme cascades, DNA fragmentation, and eventual cell blebbing and disintegration, govern programmed cell death, also known as apoptosis. In various diseases, particularly cancer, these linkages can suffer genetic alterations, underscoring the value of apoptosis detection and quantification in various research assays. Apoptosis analysis was conducted on both control and treated HeLa cells over a 24-hour period at the IC_{50} concentration of all three extracts. When HeLa cells were exposed to *C. infortunatum* for 24 hours, the decoction and aqueous extracts exhibited notable early apoptosis at their respective IC_{50} concentrations. However, the decoction extract at IC_{25} did not display significant apoptotic effects, and the hydroalcoholic extract demonstrated negligible changes in apoptosis at both IC_{50} and IC_{25} concentrations (Figure 6A, 6B).

Table 4. IC_{50} Values of *Clerodendrum infortunatum linn.* and Doxorubicin on HeLa Cell Line

Cell line	HeLa (cervical cancer) cell line			
Plant extract	Hydro alcoholic	Decoction	Aqueous	Positive control
Clerodendrum infortunatum linn.				Doxorubicin
IC_{50} (µg/ml)	569.04 ± 2.46	242.28 ± 3.30	630.03 ± 3.21	1.31 ± 0.08

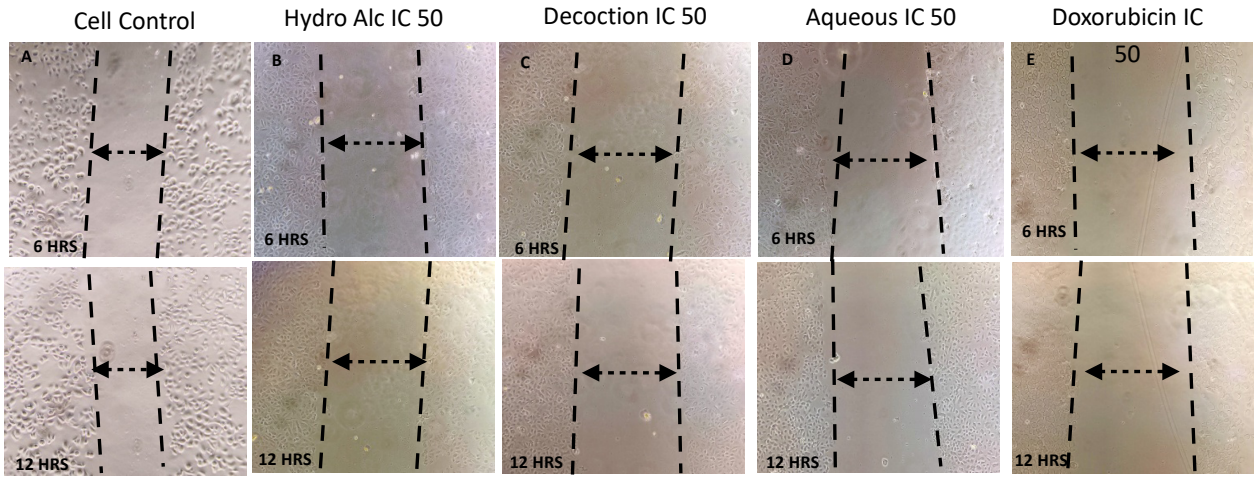

Figure 5. Scratch assay Representative Image (A). After 24 hours, HeLa cells were incubated with three extracts of *C. infortunatum* at IC_{50}. The width of scratch was examined at 0, 6, and 12 hours (OLYMPUS CKX53) at the indicated times. (100X magnification). After 6 and 12 hours, *C. infortunatum* inhibited HeLa cell migration. The first vertical group shows the control, where slight closure of wound is visible.

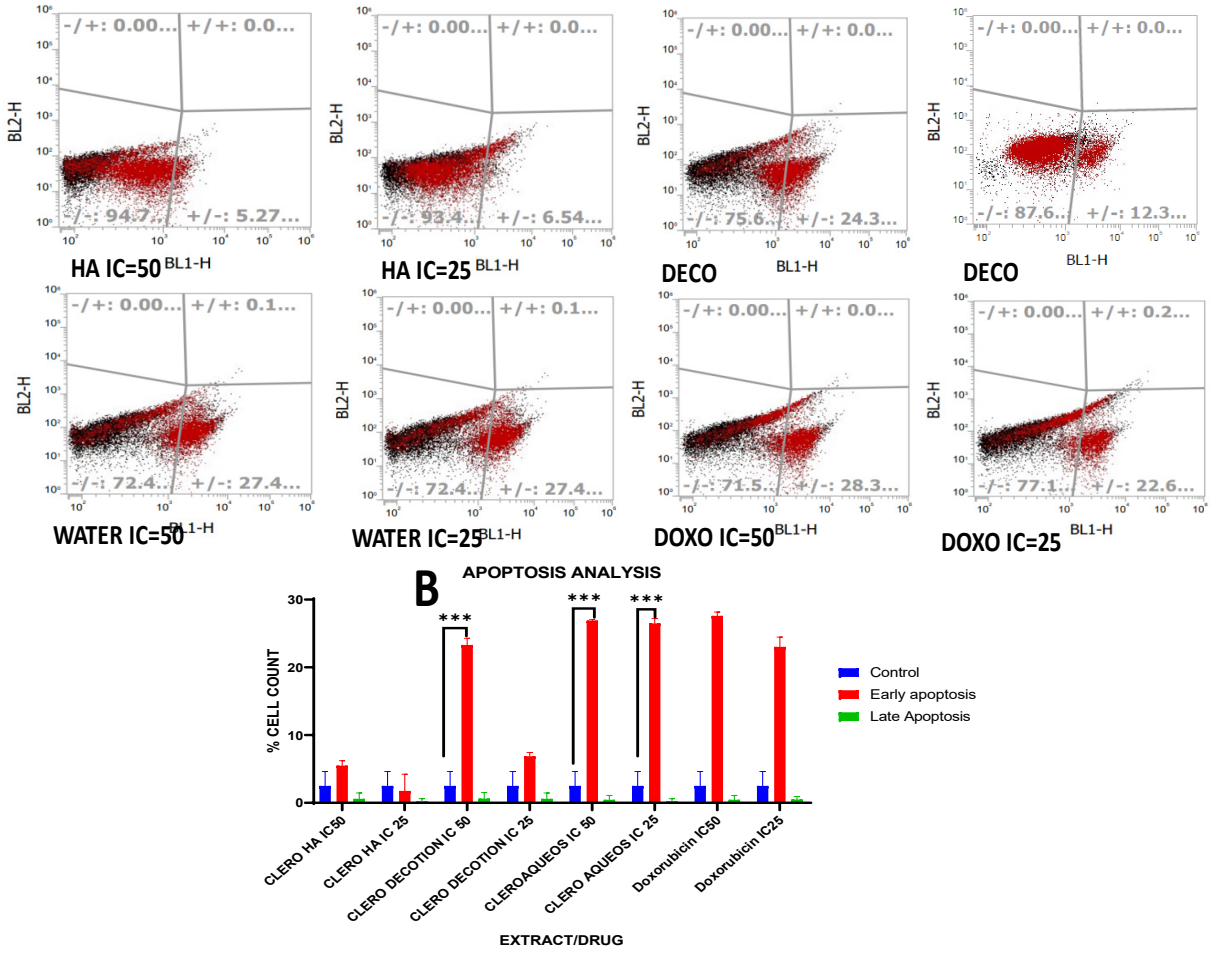

Figure 6. Assay of *C. infortunatum* -induced Apoptosis Using Flow Cytometry (A) It depicts cells treated with *C. infortunatum* at IC50 after 24 hours, and (B) Data was expressed as Mean+SD.

Similarly, DOXO induced early apoptosis at both the IC_{50} and IC_{25} concentrations after 24 hours (Figure 6A, 6B).

Assessment of C. infortunatum effect on cell cycle regulation of HeLa cells

A comprehensive cell cycle analysis was conducted over a 24-hour period, encompassing both untreated and treated HELa cells at both the IC_{50} and IC_{25} concentrations of *C. infortunatum*. The results highlighted several distinct observations. At the IC_{50} concentration of *C. infortunatum* hydroalcoholic extract, a significant arrest of the cell cycle was observed at the S-phase, whereas this effect was not pronounced at the IC_{25} concentration. *C. infortunatum* water extract at IC_{50} prompted an S-phase arrest, while at IC_{25}, a G2M phase arrest was evident. On the other hand, the decoction extract at IC50 led to a notable G2M phase arrest, while no substantial changes were seen at IC_{25}. In contrast, DOXO exhibited cell cycle arrest predominantly at the G1-phase at both IC_{25} and IC_{50} concentrations (Figure 7). Collectively, the data provided herein signifies that *C. infortunatum* holds the capability to induce apoptosis as well as hinder cell growth or obstruct the S-phase and G2M in the context of Cervical cancer (HeLa cells).

Intracellular ROS measurement

Elevated intracellular levels of reactive oxygen species (ROS) can lead to detrimental effects on cellular components. In response, cells activate defense mechanisms by upregulating the expression of antioxidants in order to counterbalance the impact of ROS. To initiate our investigation, we initially assessed the distribution of fluorescence intensity in the presence or absence of *C. infortunatum* and DOXO. Treating HeLa cells with *C. infortunatum* hydroalcoholic and water extracts at both IC_{50} and IC_{25} concentrations demonstrated a noteworthy reduction in intracellular ROS generation. Conversely, the decoction extract at IC_{50} and IC_{25} concentrations, as well as DOXO at both IC_{50} and IC_{25} concentrations, did not induce any substantial alteration in ROS production (Figure 8).

Effects of Clerodendrum infortunatum on Bax and BCL-2 expression in HeLa cells

C. infortunatum was found to induce a downregulation of the *BCL-2* gene expression. Notably, this downregulation was particularly pronounced in response to Clerodendrum aqueous and decoction extracts, as evidenced by both their IC_{50} and IC_{25} values. This pattern suggests that these extracts induce apoptosis in cancer cells primarily by inhibiting the anti-apoptotic gene *BCL-2*. However, it's important to mention that *Clerodendrum infortunatum*

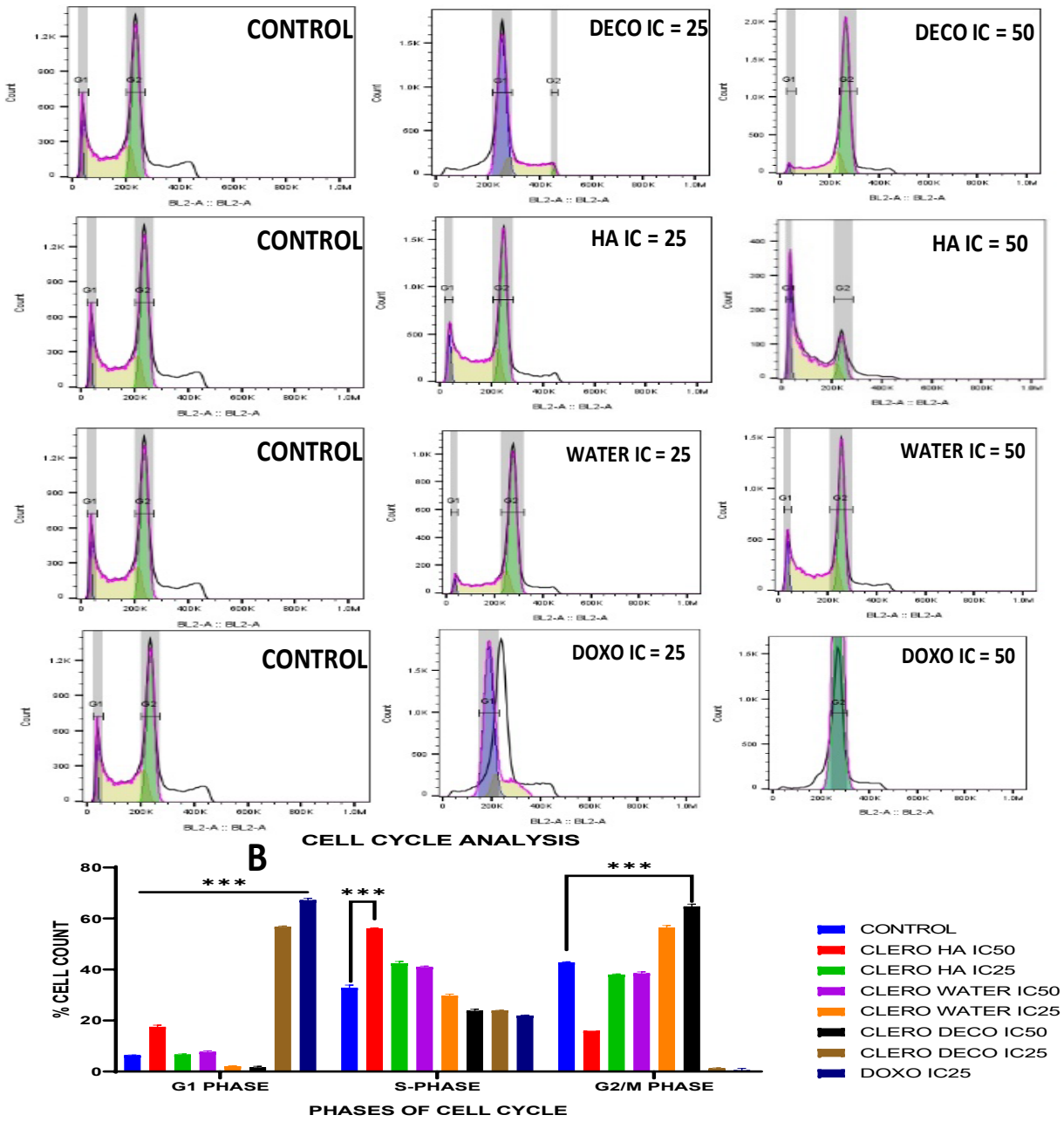

Figure 7. Flow Cytometry was Used to assess the Regulatory effect of *C. infortunatum* & DOXO on Cell Cycle Distribution in HeLa Cells. Data was expressed as Mean+SD . to compare the different phases of cell cycle for the respective concentrations of *C. infortunatum* and DOXO.

did not exhibit any significant impact on the *Bax* genes (Figure 9).

Discussion

The Clerodendrum genus has proven to be a valuable source of herbal medicinal products, particularly in developing countries where herbal remedies are widely practiced due to their perceived affordability and safety [27]. Phenolic components have garnered significant importance as potential agents for managing oxidative stress-related diseases [28, 29]. The antioxidant activity of these compounds is closely tied to the secondary metabolites present in plants and their ability to counteract unstable free radicals in the system. Very few studies have been performed on *C. infortunatum*'s species and their various solvents extract. In the present study Hydroalcoholic extract type showed noteworthy anti-oxidant property by reducing intracellular ROS production. The remarkable antioxidant properties observed in *C. infortunatum*'s leaf extract can be attributed to the high content of flavonoids and phenols.

The toxicity studies conducted on HeLa, AGS, and HT-29 cells revealed increasing cytotoxicity with rising concentrations. Prior research has highlighted the substantial *in vivo* antitumor potential of *Clerodendrum serratum* 's methanolic extract against Dalton's Lymphoma Ascites, showcasing the potential of Clerodendrum genus extracts in cancer therapy [30]. Other plants comprising flavonoids like *Tragopogon porrifolius, Cassia acutifolia,*

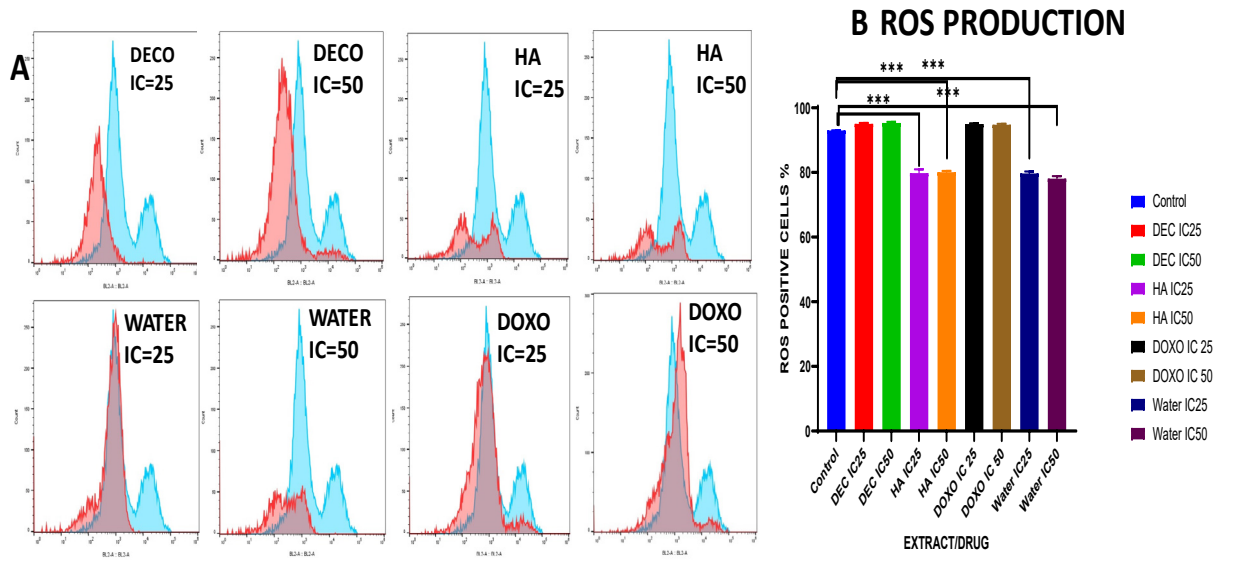

Figure 8. Effect of *C. infortunatum* on Intracellular ROS Production. Data was expressed as Mean+SD and compared to production of intracellular ROS for the respective concentrations of *C. infortunatum* and DOXO.

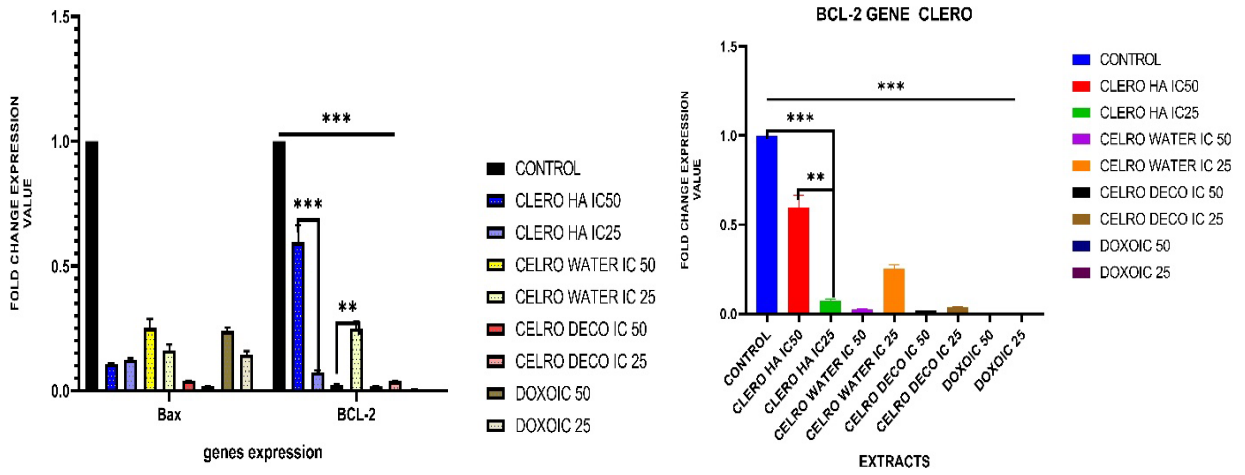

Figure 9. The mRNA Expression levels of HeLa Cells [Real time qRT-PCR]. Comprehensive analysis of Bax, and BCL-2 genes in HeLa cells treated with *C. infortunatum* and DOXO at IC_{50} and IC_{25} with medium only. B-actin gene is used as control. The results were subjected to "Two-way ANOVA" followed by Tukey Post hoc tests" at ****p<0.0001, ***p<0.001, **p<0.01 to determine the statistical significance between the groups as compared to control of respective time point

Oroxylum indicum, Rhazya stricta and Trigonella foenum-graecum have also demonstrated antioxidant and anticancer activities [31, 32]. It's important to note that the percentage yield of extracts can vary among plant parts and species, and it's influenced by the choice of solvent systems. In this study, we observed that different types of extracts showed varied types of actions accordingly, where the decoction extract exhibited higher cytotoxicity against the cervical cancer cell line (HeLa) compared to other extracts, as evident from the assay results.

In addition, there have been studies indicating that *C. infortunatum* exhibits significant antitumor activity against DLA and EAC cell lines in vitro and in vivo [33]. HPLC analysis confirmed the presence of rutin and quercetin, both of which have demonstrated anti-cancer activity in various contexts [34,35]. Rutin has been found to impact Bax and Bcl-2 expression, leading to caspase-triggered apoptosis in colon cancer cells, while also modulating AP-1 and NF-κB transactivation [36]. Quercetin is known to induce DNA interactions, trigger cell cycle arrest, and activate apoptosis in different cancer cell types [37, 38]. The presence of these compounds, along with gallic acid, likely contributes to the observed anti-cancer activity. In the present study we found quantitative presence of Rutin, Quercetin and Gallic acid in different solvent extracts. *C. infortunatum* mainly decoction and aqueous type showed impact on BCL-2 by reducing its expression in the present study.

C. infortunatum water and decoction extracts showed predominantly apoptosis and cell cycle arrest, whereas HA extract showed good anti-oxidant activity. These extract related variations need to be validated by further studies

in detail. Delving into the molecular-level mechanisms of its action could prove essential for formulating dosages in the future.

In conclusion, the results of our study highlight the potential of *C. infortunatum linn.* as a promising source of active compounds with efficacy against cervical cancer cells. Our investigation suggests that the anti-tumor activity of *C. infortunatum* could likely be attributed to the presence of flavonoids like rutin and quercetin. These compounds are believed to contribute significantly to its therapeutic effects. Given these findings, *C. infortunatum* holds promise for further exploration as a potential anti-cancer agent. Further research and studies are warranted to comprehensively understand its mode of action and to develop it as a viable candidate for cancer treatment.

Author Contribution Statement

CRediT authorship contribution statement

Jayshree Changade: Conceptualization, Software, Formal analysis, Writing - original daft. Harsha Thanvi: Data curation, Formal analysis, Methodology. Chandrashekhar Raut: Supervision, Writing - review & editing. Manasi V. Chavan: Methodology, Data curation, Prachi V. Prasad: Methodology, Data curation, Vaibhav Ladke: Formal analysis, Software, Investigation, Validation, Writing original daft. All authors approved the submitted manuscript

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Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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Declaration of competing interest

All authors declare that they have no known competing financial interests.

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