



Calculus bovis Ekstraktının Antioksidan, Sitotoksik, Apoptotik/Nekrotik ve Gen Ekspresyon Düzeylerinin Belirlenmesi

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Kanser, her yıl milyonlarca insanın ölümüne neden olmakta ve tüm insanlığı etkilemektedir. 2030 yılına kadar 22 milyon yeni vaka öngörülmektedir. Standart kemoterapötik ajanların yan etkileri ve tedavi sonrası tümör tekrarı, doğal ve yenilebilir ürünlerden elde edilen moleküllerin geliştirilmesine yönlendirmiştir. Bu çalışmada, *Calculus bovis* ekstraktlarının kanser hücreleri üzerindeki etkileri incelenmiştir. MDA-MB-231, A549, PANC-1 ve OVCAR-3 hücre hatlarında MTT testi ile sitotoksik aktivite değerlendirilmiştir. Apoptotik/nekrotik etki çift boyama yöntemi ile analiz edilmiştir. *Bax*, *Bcl-2*, *p21* ve *p53* gen ekspresyon seviyeleri OVCAR-3 hücre hattında metanol ekstraktında qRT-PCR yöntemiyle ölçülmüştür. Elde edilen sonuçlar gösteriyor ki aseton ekstraktı, OVCAR-3 hücre hattında belirgin sitotoksik etki göstermiştir. Aynı hücre hattında metanol ekstraktında *Bcl-2* gen ekspresyonunun baskılandığı ve apoptozu tetiklediği belirlenmiştir. Bulgular, *Calculus bovis*'in özellikle over ve meme kanseri tedavisi için potansiyel bir kemoterapötik ajan olabileceğini göstermektedir. Ekstraktın farklı kanser hücre hatlarına karşı yan etki göstermeden etkili olacağını umuyoruz. Ancak, yan etki profili henüz belirlenmediğinden araştırması gerekmektedir.

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Determination of Antioxidant, Cytotoxic, Apoptotic/Necrotic and Gene Expression Levels of *Calculus bovis* Extract

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ABSTRACT

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Cancer kills millions of people every year and affects all of humanity. 22 million new cases are projected by 2030. Side effects of standard chemotherapeutic agents and tumor recurrence after treatment have led to the development of molecules derived from natural and edible products. In this study, the impact of *Calculus bovis* extracts on cancer cells was

Key words:

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examined. In MDA-MB-231, A549, PANC-1, and OVCAR-3 cell lines, cytotoxic activity was evaluated by MTT assay. The apoptotic/necrotic effect was analyzed by the double staining method. *Bax*, *Bcl-2*, *p21*, and *p53* gene expression levels were measured by qRT-PCR in methanol extract in the OVCAR-3 cell line. The results showed that acetone extract showed a significant cytotoxic effect in the OVCAR-3 cell line. In the same cell line, the methanol extract suppressed *Bcl-2* gene expression and triggered apoptosis. The findings suggest that *Calculus bovis* may be a potential chemotherapeutic agent, especially for the treatment of ovarian and breast cancer. We expect the extract to be effective against different cancer cell lines without side effects. However, its side effect profile is yet to be determined and needs further research.

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1. INTRODUCTION

Cancer encompasses various illnesses characterized by uncontrolled cellular growth, replicative immortality, and resistance to programmed cell death. The primary cause is damage or mutation of tumour suppressor genes that inhibit apoptosis or regulate signals that promote cell growth (Pérez-Herrero and Fernández-Medarde, 2015; Sung et al., 2021). Cancer is the second leading cause of death globally, following cardiovascular diseases (Ergin et al., 2019; Sung et al., 2021). In 2022, approximately 20 million new cancer cases were reported worldwide and 9.7 million people died from cancer (Bray et al., 2024). Statistics predict that the number of new cases will reach 22 million by 2030 (Ergin et al., 2019).

Unchecked cell proliferation and the suppression of signals initiating cell death are pivotal in cancer progression (Tor et al., 2014). Apoptosis, a form of programmed cell death, is a crucial mechanism for eliminating cancer cells. Apoptosis can inhibit metastatic spread by killing irregularly placed cells (Su et al., 2015). It selectively targets cancerous cells without affecting neighboring tissues or causing inflammation (Kaya et al., 2015; Wong, 2011). In addition, it regulates cellular suicide without causing an inflammatory response, underlining its importance in cancer (Su et al., 2015).

Numerous elements have been documented to play a part in controlling apoptotic processes. Factors affecting apoptosis through any mechanism also impact the prognosis of cancer patients. Key genes like *Bax*, *Bcl-2*, *p53*, and *p21* are involved in the apoptosis pathway and significantly impact cancer prognosis (Su et al., 2015). *p53* is a gene involved in numerous cellular processes and influences how apoptosis is controlled (Aubrey et al., 2018). Its ability to initiate transcription suggests that the genes it activates are vital for its tumor-suppressing function. In this context, *p53*'s capacity to initiate transcription suggests that the genes it induces might play a pivotal role in fulfilling its function as a tumor suppressor. Ongoing research aims to understand the diverse genes involved in *p53*-mediated apoptosis (Coleman et al., 2017). Although many stimuli and conditions can trigger apoptosis, not all cells respond similarly. Therefore, current studies focus on understanding the cell cycle mechanisms and specific stimuli that trigger apoptosis, as well as the signaling pathways involved (Elmore, 2007). To this end, the field of apoptosis research is advancing rapidly. These studies will gain a new and different dimension for cancer treatment.

Cancer treatment faces numerous challenges, including adverse side effects, tumor recurrence, and the need for surgical interventions (Haileselassie et al., 2019; Xavier and Palmeira, 2024). Research into cytotoxic agents has led to discovering new therapeutic compounds, with many anticancer drugs derived from natural resources like plants and animals (Luca et al., 2018). About 60% of authorized anticancer medicines currently consist of natural materials (Man et al., 2012).

Recently, steroidal compounds with anticancer and antibacterial properties have gained attention. Ox gallstones, or "*Calculus bovis*," are valuable due to their rarity and therapeutic components, such as cholic acid (CA) and deoxycholic acid (DCA), which have potent anticancer and antibacterial effects (Huang et al., 2024; Kong et al., 2010; Zang et al., 2011; Zhao, 2024). Despite their historical use in East Asian medicine, research on the anticancer mechanisms of *C. bovis* extracts is limited. Due to the scarcity and high cost of natural *C. bovis*, studies are usually conducted with artificially prepared *C. bovis sativus* (CBS) (He et al., 2017; Peng et al., 2014; Xiang et al., 2017; Yu et al., 2020; Zang et al., 2011). *C. bovis* has been used clinically for 2000 years in East Asian countries, especially in China, for various diseases such as cirrhosis, hepatitis, epilepsy, coma, jaundice, and digestive system disorders (Wang and Carey, 2014; Zang et al., 2011).

The aim of this study was to characterize the biological effects of *C. bovis* extract in human cancer models. Concentration-dependent cytotoxicity was assessed in human breast cancer (MDA-MB-231), lung cancer (A549), pancreatic cancer (PANC-1), and ovarian cancer (OVCAR-3) cell lines to determine the extract's effect on cell viability. The mode of cell death was further characterized by double staining in MDA-MB-231, A549, and OVCAR-3 cells, while in OVCAR-3 cells, apoptosis induction, associated cell death pathways, and the expression levels of apoptosis-related genes (*Bax*, *Bcl-2*, *p53*, *p21*) were examined. In addition to these analyses, the antioxidant activity of the methanol extract was evaluated using the DPPH radical scavenging assay.

2. METHODOLOGY

2.1. Obtaining The Material

C. bovis was obtained naturally from Malet slaughterhouse in Malatya, dried, weighed 1 g, ground, and dissolved in 10 mL each of acetone, ethanol, and methanol. Polar solvents allow the polyphenols contained in plants to be extracted effectively. In this sense, acetone, ethanol, and methanol solvents were preferred in the extraction process because they are polar solvents (Yalçın et al., 2017). The extracts were incubated in a shaking oven (Nüleon NCI55) at 37°C for 72 hours. After filtration through Whatman No. 1 filter paper, the extracts were evaporated in a rotary evaporator at 40°C for four-six hours. The extracts were dissolved in DMSO (Dimethyl Sulfoxide) and stored at +4°C. Dilutions were prepared with medium. DMSO has a wide range of solvents, soluble with both polar (such as water) and apolar (such as hydrocarbons) solvents; it is soluble with water and can also form hydrogen bonds with polar solvents, while with apolar solvents, it can enter into weak interactions such as Van der Waals forces. Because of these DMSO properties, it was preferred that the master stock be prepared (Kadioğlu Dalkılıç et al., 2023). Four different concentrations (500, 200, and 100 µg/mL) were then prepared in

DMEM medium, and the master stock as well as the working concentrations were repeated three times and used fresh.

2.2. Cell Lines and Cell Cultures

MDA-MB-231, A549, PANC-1, and OVCAR-3 cell lines were purchased from the American Type and Culture Collection (ATCC) acquired. These four cell lines were cultured in 25 cm² flasks in DMEM (Dulbecco's Modified Eagle's Medium, High Glucose / 25 mM 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS)) enriched with 5% CO₂ at 37°C.

2.3. Assessment of Cell Viability

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability and Cell Counting Kit-8 (CCK-8) provided by MedChemExpress (USA) was used. Cells grown in 25 cm² flasks were inoculated in 96-well plates at 5x10³ cells per well and incubated for 24 hours. Three different solvents (methanol, acetone, ethanol) and three different concentrations (500, 200 and 100 µg/mL) of *C. bovis* extract were prepared and six wells were separated for each concentration. Absorbance values were measured at 570 nm wavelength with an ELISA microplate reader (KHB ST-360). This measurement was repeated five times and averaged, and % cell viability levels were calculated.

Doxorubicin (2.5 µg/mL) was used as the positive control (PC) to confirm the validity of the assay; it served solely to demonstrate assay functionality and was not intended for direct quantitative comparison with the extract concentrations, while medium alone served as the negative control (NC) (George and John A, 2006; Karakaş et al., 2017; Ulukaya et al., 2008). The percentage cell yield is expressed in equation one [1] (Shanmugapriya et al., 2019).

$$\text{Cell viability (\%)} = \frac{\text{Extracted group}}{(-)\text{Control group}} \times 100 \quad [1]$$

2.4. Evaluating Necrotic and Apoptotic Behavior

Evaluation of apoptotic and necrotic activity was conducted using dual staining with Propidium Iodide (PI) (MedChemExpress, 25535-16-4, NJ, USA) and Hoechst 33258 (H33258) (MedChemExpress, 23491-45-3, NJ, USA). MDA-MB-231, OVCAR-3, and A549 cell lines were seeded on sterile coverslips in a 6-well plate with 10,000 cells in 100 µL and incubated for 4-5 hours to adhere. Two milliliters of DMEM were added, and cells were incubated for 24 hours at 37°C with 5% CO₂. Subsequent to incubation, the medium in the wells was aspirated, and swapped out with a medium that included 2 mL of *C. bovis* extract at maximum concentration (500 µg/mL) after which it was incubated for 48 hours. Then, 1 mL of a solution containing H33258 (5-10 µg/mL) and PI (1 µg/mL) in PBS was added after removing 1 mL of medium. The plate was incubated in the dark for 30 minutes and evaluated by EVOS FLc Fluorescence Inverted Microscopy (Karakaş, 2013). After incubation, the morphology of the cells was compared with the control group (negative: untreated cells; positive: 2.5 µg/mL Doxorubicin). Doxorubicin was used as the confirm the validity of the assay; it served solely to demonstrate assay functionality. In addition to all this, considering the lower sensitivity of fluorescence-

based assays compared to cell viability assays, extracts were used at high concentrations to obtain measurable apoptotic and necrotic responses. To validate the functionality of the assay, doxorubicin was included as a positive control without direct dose comparison with the extracts.

2.5. Assessing Antioxidant Activity

The antioxidant potential of methanol extract of *C. bovis* at 100, 50, 25, and 12.5 mg/mL was assessed using the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging assay. These solutions were prepared from the lyophilized drug. Ascorbic acid served as the PC, methanol as the NC. After incubation in darkness for 30 minutes, absorbance was measured at 517 nm to calculate percent inhibition. The measurement was performed with the help of an ELISA (Enzyme-Linked Immuno Sorbent Assay) microplate reader, and the indicated formula was used to determine the scavenging rate of DPPH radicals obtained [2] (Dhanani et al., 2013). Three duplicates of each concentration were tested.

$$\text{Antioxidant activity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control group}} \times 100 \quad [2]$$

2.6. Performing the Quantitative real-time PCR (qRT-PCR) Reaction

RNAs were isolated with the Geneaid RNA Mini Kit (Geneaid-ISO 9001). This was followed by cDNA synthesis, carried out using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Lot 00754387, Lithuania) protocol. 100 ng RNA (30 µL) was heated at 25°C for 10 minutes, incubated at 37°C for 120 minutes, followed by a denaturing step at 85°C for 5 minutes in an Applied Biosystems SimpliAmp Thermal Cycler. At the end of the process, the resulting total cDNA product was diluted to 40 µL. *Bax*, *Bcl-2*, *p21*, and *p53* gene expression was also quantified by qRT-PCR using SYBR Green master mix (AM02-020). Analysis was performed using the StepOnePlus® Real-Time PCR System and a reaction volume of 10 µL as recommended. Cycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 5 s, 60 °C for 10 s and 72° C for 20 s). The quality of PCR products was assessed by agarose gel electrophoresis (Dalkılıç et al., 2022). The primers used are given in Table 1. *GAPDH* served as the internal control for data normalization. Evaluation of gene expression profiles was analyzed using the $2^{-\Delta\Delta CT}$ method, and data are expressed as mean fold changes $\pm$ SEM for three independent amplifications (Livak and Schmittgen, 2001). Based on the expression data, the *Bax/Bcl-2* ratio was used to assess apoptosis and cell death (Mai et al., 2020).

Table 1: Primer sequences for qRT-PCR

Gene name	Primer sequences	
<i>Bcl-2</i>	Forward (5' to 3')	GAA GGT TTC CTC GTC CCT GG
	Reverse (5' to 3')	CTG TGT TGA AAC AGG CCA CG
<i>Bax</i>	Forward (5' to 3')	CCC CGA TTC ATC TAC CCT GC
	Reverse (5' to 3')	GAG CTA GGG TCA GAG GGT CA
<i>p21</i>	Forward (5' to 3')	GCT TCA TGC CAG CTA CTT CC
	Reverse (5' to 3')	CCC TTC AAA GTG CCA TCT GT
<i>p53</i>	Forward (5' to 3')	GCT GCT CAG ATA GCG ATG GTC T
	Reverse (5' to 3')	CAT CCA AAT ACT CCA CAC GCA A
<i>GAPDH</i>	Forward (5' to 3')	GAA GGT GAA GGT CGG AGT C

Reverse (5' to 3')

GAA GAT GGT GAT GGG ATT TC

2.7. Analysis

Data obtained from cytotoxicity assays were statistically analyzed using one-way analysis of variance (ANOVA) and evaluated at a significance level of $p < 0.05$. Expression data were analyzed using statistical analysis, including ANOVA and Student's t-test, and significance was set at $p < 0.005$. The SPSS statistical program for Windows (Version 22, SPSS Inc., Chicago, IL, USA) was used to perform these statistical procedures. DataAssist (version v3.01) and GraphPad Prism (version 8.0.2) software were used to graph the data.

3. RESULTS

This study aims to systematically investigate and evaluate the apoptotic effects of *C. bovis* extract on various cancer cell lines through different biochemical and cellular assays. In this sense, the cytotoxic effects of acetone, ethanol, and methanol extracts of *C. bovis* on MDA-MB-231, A549, PANC-1, and OVCAR-3 cell lines were examined, and apoptotic and necrotic activities were determined in these cell lines. In addition, antioxidant effects were evaluated with methanol extract, and effects on the apoptotic pathway were observed by analyzing *Bcl-2*, *Bax*, *p21*, and *p53* gene expression levels.

3.1. Cytotoxic activity

In this study, the cytotoxicity of *C. bovis* was evaluated on these cell lines using the MTT assay. Notable cytotoxic effects were noted in OVCAR-3 and MDA-MB-231 cells at a 500 $\mu\text{g/mL}$ concentration in acetone extract (Figure 1).

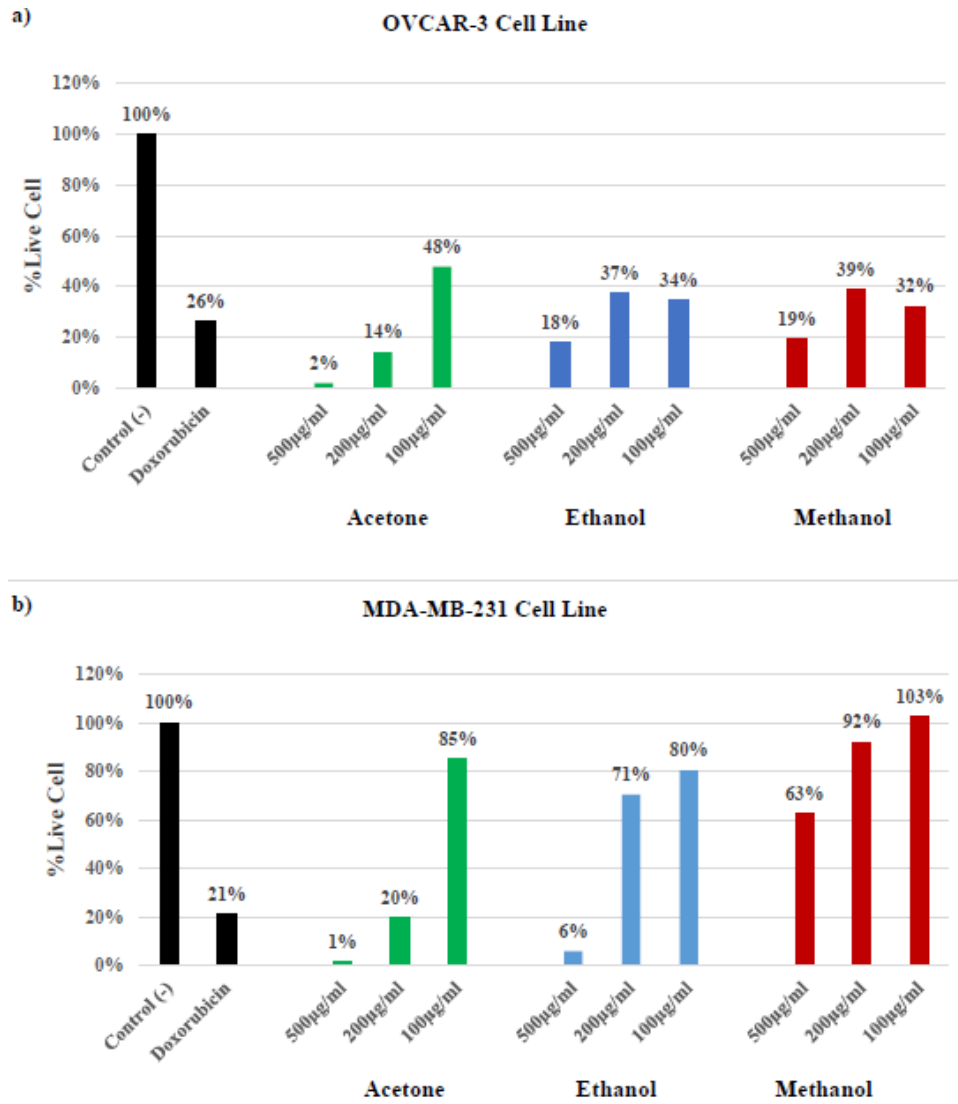


Figure 1. MTT assay result of *C.bovis* extracts in (a) OVCAR-3 (Human ovarian cancer) and (b) MDA-MB-231 (Human breast cancer) cell lines expressed as % viable cells. Cytotoxic effect of *C. bovis* on the OVCAR-3 (Human ovarian cancer) cell line. *((-) Control: DMEM; (+) Control: Doxorubicin (used only to confirm assay validity, not for direct dose comparison); Measurement result of different concentrations of *C. bovis* 500, 200, 100 µg/mL)

In particular, a significant cytotoxic effect on OVCAR-3 cells was observed for all three solvent extracts. The acetone extract showed 2% cell viability at 500 µg/mL, 14% at 200 µg/mL, and 48% at 100 µg/mL, demonstrating a clear dose-dependent effect. However, for the ethanol and methanol extracts, cell viability at 200 µg/mL was slightly higher than at 100 µg/mL, indicating a non-linear dose-response relationship. Overall, the highest cytotoxicity was observed at 500 µg/mL for all extracts, confirming their anti-ovarian cancer activity (Figure 1a).

In the MDA-MB-231 cancer cell line, 1% survival was observed at 500 µg/mL, while 20% viability was observed at 200 µg/mL. In the ethanol extract, only 6% survival was observed at a 500 µg/mL concentration. No significant cytotoxicity was observed when other concentrations were examined against the MDA-MB-231 cell line. An overall evaluation of the OVCAR-3 and MDA-MB-231 cell lines shows that cytotoxicity decreased dose-dependently (Figure 1b).

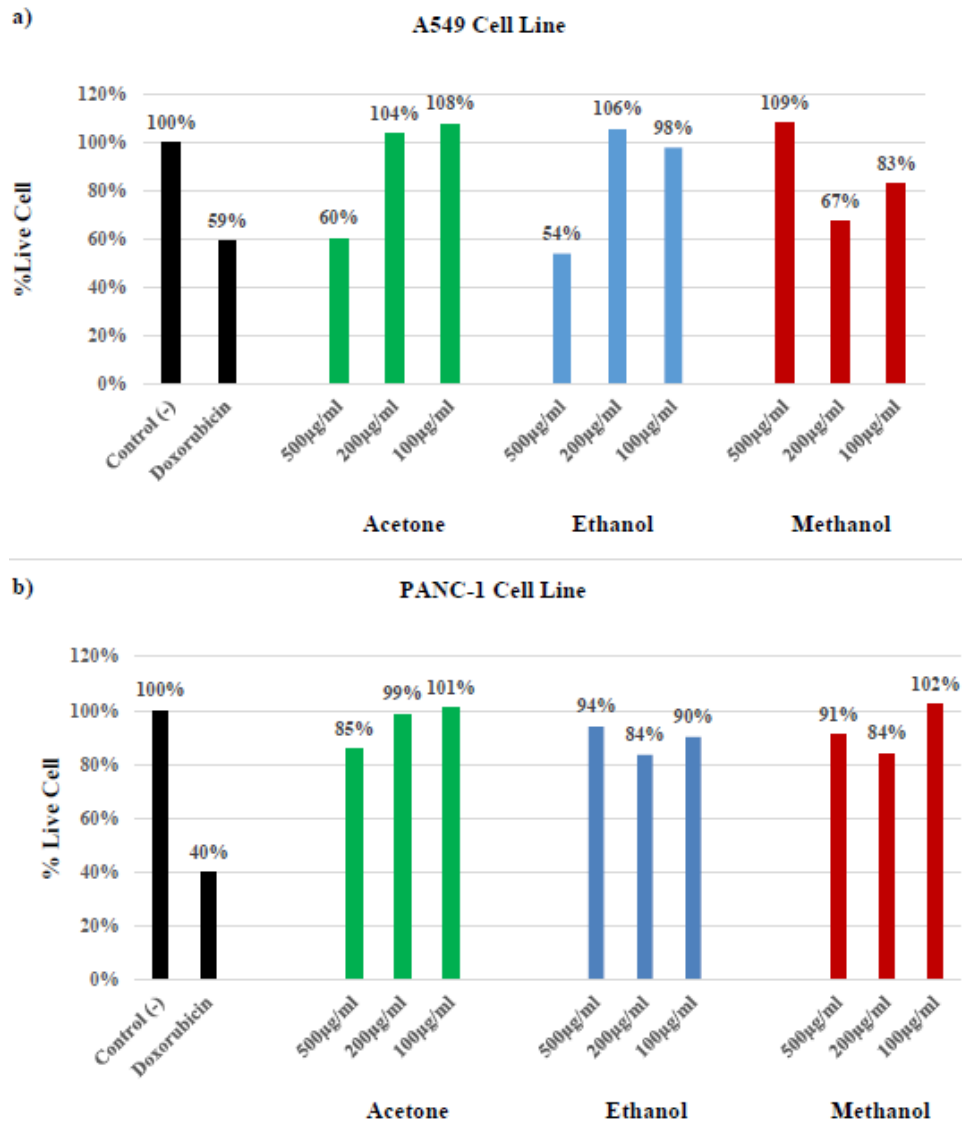


Figure 2. Cytotoxic effect of *C. bovis* on A549 (human lung cancer) and PANC-1 (Human pancreatic cancer) cell lines. *((-) Control: DMEM; (+) Control: Doxorubicin (used only to confirm assay validity, not for direct dose comparison); Measurement result of different concentrations of *C. bovis* 500, 200, 100 µg/mL)

When the cytotoxicity in the A549 cell line was examined, the ethanol extract at a concentration of 500 µg/mL showed the best effect (54% survival), while no significant cytotoxicity was observed at other concentrations (Figure 2a). While survival of 84% was observed in the ethanol extract at a concentration of 200 µg/mL on the PANC-1 cell line, it was determined that the effect on this cell line, in general, remained minimal (Figure 2b).

Table 2. OVCAR-3 and MDA-MB-231 cell lines IC₅₀ results

	OVCAR-3 cells			MDA-MB-231 cells		
	Methanol	Acetone	Ethanol	Methanol	Acetone	Ethanol
IC ₅₀	448 µg/mL	161 µg/mL	427 µg/mL	223 µg/mL	169 µg/mL	235 µg/mL

* OVCAR-3: Human Ovarian Cancer Cell Line, MDA-MB-231: Human Breast Adenocarcinoma Cancer Cell Line, IC₅₀: Half maximal inhibitory concentration

Table 2 reports the IC₅₀ values of methanol, acetone, and ethanol extracts for OVCAR-3 and MDA-MB-231 cell lines. These values represent the concentrations required to inhibit 50% of cell growth.

Since inhibition did not exceed 50% in PANC-1 and A549 cell lines, IC_{50} values could not be determined. The acetone extract exhibited lower IC_{50} values compared to the other extracts, with 161 $\mu\text{g/mL}$ for OVCAR-3 and 169 $\mu\text{g/mL}$ for MDA-MB-231. However, the fact that all IC_{50} values were above 100 $\mu\text{g/mL}$ indicates that the extracts do not demonstrate strong cytotoxic activity. Nevertheless, the acetone extract in particular suggests a relatively higher cytotoxic potential compared to the other solvents.

When a general evaluation is made, the OVCAR-3 cell line showed significant cytotoxic activity in all solvents, and effective cytotoxicity was observed in the MDA-MB-231 cell line. Unfortunately, the same was not true for A549 and PANC-1 cell lines, which did not show significant cytotoxicity. A statistical analysis of cytotoxic activity was performed using a one-way ANOVA test with SPSS version 22. In the light of the data obtained, significance was found only on the OVCAR-3 cell line with $p < 0.044$. In the other three cell lines, cytotoxic activity was not considered significant since it was greater than $p < 0.05$.

3.2. Determination of Apoptotic/Necrotic Activity and Dual Staining Results

The results of double staining to assess the effects of solvents on cell lines showed apoptotic and necrotic effects that varied depending on the solvent type and cell type. In staining, Hoechst labeled chromatin condensation (red arrows) and apoptosis (white arrows) with blue fluorescence, while PI labeled necrotic cells with impaired membrane integrity (green and orange arrows) with red fluorescence. The corresponding results are expressed on Figure 3. Moreover, the lack of staining with PI (red dye) indicates that these cells undergo apoptosis. In contrast, necrotic cells, which cease metabolic and membrane activities but retain their morphology, typically stain positive with both dyes, with PI being distinctive for necrotic cells. The findings are essential to explain the cytotoxic effects of different solvents (Jabs et al., 2017). In this study, the effects of *C. bovis* on A549, MDA-MB-231, and OVCAR-3 cell lines were evaluated by using acetone, ethanol, and methanol extractions, and apoptotic and necrotic effects were assessed with H33258 and PI stains. Staining results indicate the presence of cell type-specific altered responses.

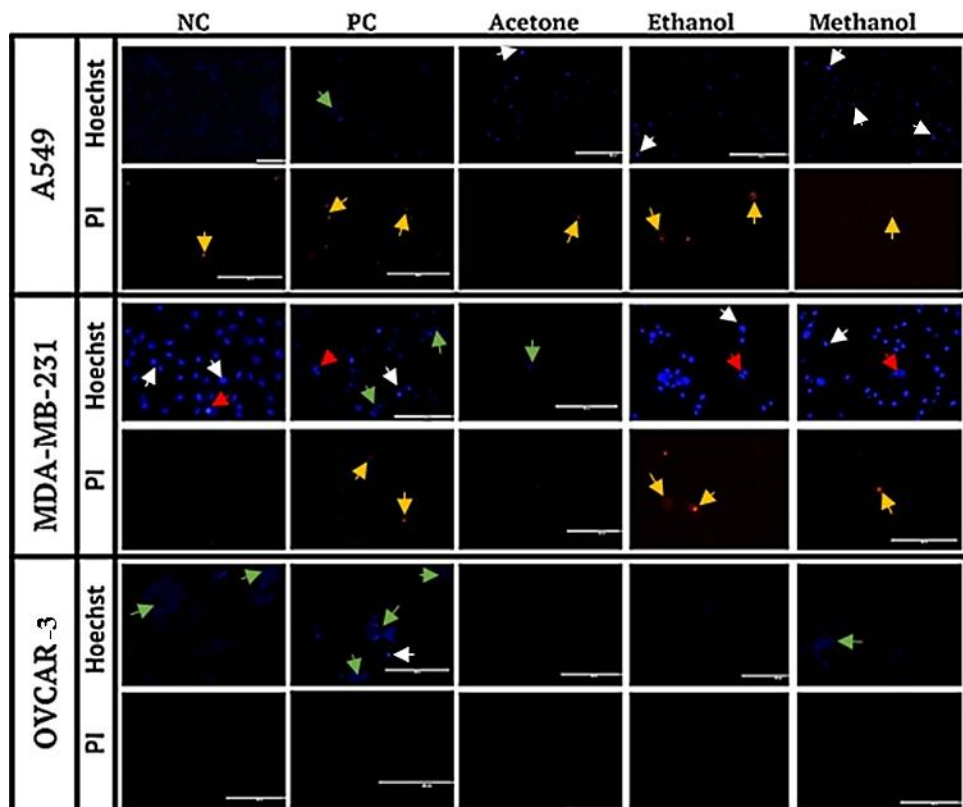


Figure 3. Detection of dual staining apoptotic/necrotic activity of *C. bovis* in A549, MDA-MB-231 and OVCAR-3 cell lines. *Images were taken with a Fluorescence Inverted Microscope at 20x magnification. Orange arrows indicate necrosis, white arrows indicate pyknosis, green arrows indicate fragmented nuclei, and red arrows indicate heterochromia (PC = Positive control (Doxorubicin 2.5 $\mu\text{g/mL}$), NC = Negative control (untreated cells))

The dual staining experiment results were concordant with MTT assay and showed a markedly reduced number of cells. The NC group (untreated cells) were viable and proliferated in all cancer cell lines. H33258 (blue) dye effectively stained the cells in this group, showing intact membranes and healthy nuclei (indicated by white arrows in Figure 3). The PI stain did not stain these cells, indicating that membrane integrity was intact and necrosis was not indicated. As a result, these cells maintained a viability and proliferation pattern consistent with anticipation. In contrast, the PC group with doxorubicin treatment showed necrosis that was fatal to cells. This was reflected in a reduced number of cells and density and PI dye's staining of the PC group (indicated by orange arrows). Figure 3 illustrates the experiment's outcomes aimed at understanding the mechanisms underlying this reduction in cell count.

The H33258 stain observed distinct blue signals in A549 and MDA-MB-231 cell lines. This indicates that ethanol induces apoptotic cell death in these cell lines. Bright blue intensities of the H33258 stain support chromatin condensation and the efficiency of the apoptotic process. As seen in Figure 3, white arrows also indicate the presence of apoptosis. In contrast, the limited red signals of the PI stain indicate that cell death is mediated by apoptosis rather than necrosis. The predominance of apoptosis in these two cell lines suggests that the effect of ethanol on these cells leads to controlled cell death (Figure 3).

Acetone treatment showed a moderate apoptotic effect in A549 and MDA-MB-231 cells, but the necrotic effect remained minimal. In OVCAR-3 cells, apoptotic and necrotic signs were almost absent, suggesting that this cell line is resistant to this solvent. These findings indicate that acetone may produce different toxic effects depending on cell type (Figure 3).

Methanol, on the other hand, showed a generally low toxicity profile. Limited apoptotic effects were observed mainly in A549 and MDA-MB-231 cells, while the effects of methanol were minimal in OVCAR-3 cells. This suggests that methanol may have a low capacity to penetrate the cell membrane or a limited potential to activate cellular death mechanisms (Figure 3).

Overall, the cytotoxic effects of used solvents were dependent on cell type, chemical properties, and mechanisms of action on cellular systems. In particularly, A549 and MDA-MB-231 cells displayed a more sensitive profile, as opposed to OVCAR-3 cells which were more resistant to solvents, most notably ethanol and acetone. These disparities suggest that we need to do a lot more work at the molecular level and that it is essential to know how solvents influence biological systems. Furthermore, a detailed examination of the effects of solvents on apoptotic and necrotic mechanisms will contribute to determining the safety profiles of these substances in biological uses.

3.3. Antioxidant Activity Results

Antioxidant effects were evaluated using the DPPH radical scavenging capacity method using *C. bovis* methanol extract. When the results obtained were assessed, while the methanol extract of *C. bovis* was 51% at a concentration of 100 µg/mL, 75% was obtained at 12.5 µg/mL. As a result of the determination of the antioxidant effect, it was observed that there was no antioxidant effect and a dose-dependent inverse proportion. In other words, it was concluded that there was an effect in the direction of decreasing antioxidant effect as the dose increased (Table 3). However, statistical analysis revealed that these differences were not significant ($p>0.05$). This finding suggests that the extract did not exhibit a substantial change in antioxidant activity within the tested concentration range.

Table 3. Antioxidant effect of *C. bovis* methanol solvent by DPPH radical scavenging capacity method

Concentrations	12,5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	Positive Control (Ascorbic acid)
Sample	77±30.3	42.5 ± 37.5	49 ± 31	51 ± 28	80

3.4. qRT-PCR Analysis Results

In order to determine the molecular mechanism of the cytotoxic action, the expression levels of genes involved in the apoptosis mechanism were quantitatively measured by qRT-PCR. In line with the data obtained from cytotoxicity experiments, gene expression analysis was continued in this cell line since the highest effect was observed in the OVCAR-3 cell line. The OVCAR-3 cell line was treated with *C. bovis* methanol extract at a 500 µg/mL concentration for 48 hours. In gene expression analysis,

the levels of *Bax*, *Bcl-2*, *p53*, and *p21* genes were evaluated and normalized to the *GAPDH* gene. The data obtained are presented in detail in Figure 4.

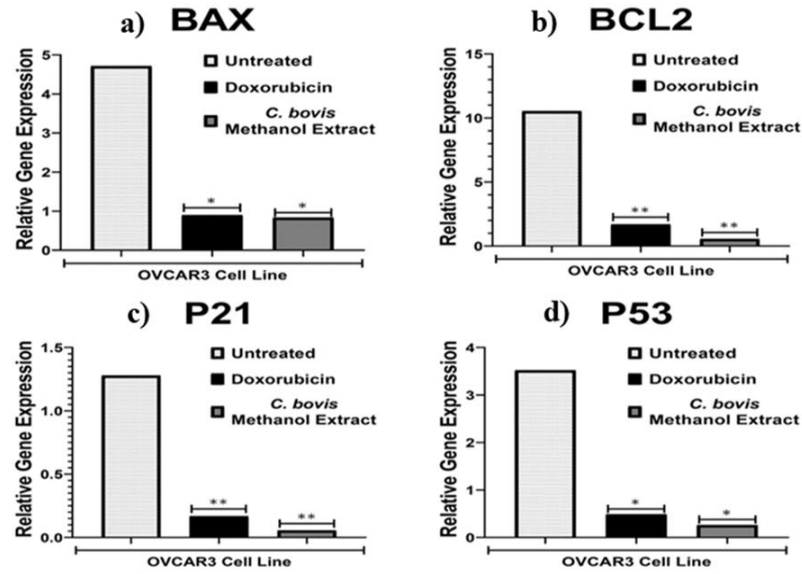


Figure 4. Quantitative real-time PCR was used to evaluate the effects of *C. bovis* extract on the expression of (a) *Bax*, (b) *Bcl2*, (c) *p21*, and (d) *p53* genes. *In the OVCAR-3 cell line, the relative gene expression in treated cells was compared with untreated cells at concentrations treated with an extract for 48 hours. Doxorubicin was used as a positive control. Expression was normalized relative to endogenous control; *GAPDH* gene expression. Significant difference was analyzed by one-way ANOVA test, where: ** $p < 0.0001$, * $p < 0.001$ compared to untreated cells. The bars represent the mean $\pm$ SD.

With the widespread use of the anticancer drug doxorubicin, PC was used in all expressions. These data indicated lower expression levels of these genes compared to the control group, demonstrating that doxorubicin, a potent chemotherapeutic agent, reduces their expression (Tacar et al., 2013). The results show that both *C. bovis* methanol extract and doxorubicin significantly reduced *Bax* and *Bcl-2* gene expressions ($p < 0.001$ and $p < 0.0001$). This decrease in expression levels was overcome by doxorubicin, which was used as a positive control for all genes (Figure 4).

The *Bax/Bcl-2* ratio is known to play a critical role in apoptosis induction in many cancer types. In this context, according to our results, the untreated group was more prone to apoptosis with a high *Bax/Bcl-2* ratio (0.741), indicating a high probability of apoptosis initiation in cells. On the other hand, Doxorubicin treatment exhibited a protective effect against apoptosis by reducing the *Bax/Bcl-2* ratio to 0.343, and similarly, *C. bovis* treatment may have prevented apoptosis by reducing the *Bax/Bcl-2* ratio to 0.41. While the untreated group was prone to apoptosis, doxorubicin and *C. bovis* treatments inhibited the onset of apoptosis and protected the cell from apoptosis.

4. DISCUSSION and CONCLUSIONS

In recent years, there has been a growing global interest in drugs of plant and animal origin, especially in the evaluation of new compounds for their potential cytotoxic activity in cancer research.

Unlike conventional chemotherapeutic drugs, which aim to stop proliferation and induce cell death, natural compounds offer promising alternatives with less potential for side effects. This drives ongoing research into traditional remedies, highlighting their significance in cancer treatment and prevention on a global scale (Demir et al., 2019). Notably, over 60% of newly developed anti-cancer medications derive from natural products or their derivatives, underscoring their therapeutic potential and the need for continued exploration in this field (Abdullah et al., 2014).

In this study assessed the antioxidant and cytotoxic effects of three *C. bovis* extracts on A549, MDA-MB-231, and OVCAR-3 cell lines, as well as their impact on cell death patterns and gene expressions in OVCAR-3 cells. For cytotoxicity testing the MTT assay was used, and dual staining with H33258 and PI assessed mitochondrial function and DNA binding activities in the tested cell lines (Plengsuriyakarn et al., 2012). Doxorubicin was included as a positive control to confirm the validity of the cytotoxicity and apoptosis assays. It should be emphasized that its role was solely to verify assay functionality and not to provide a direct quantitative comparison with the *C. bovis* extracts. Furthermore, relatively high extract concentrations were applied in apoptosis and gene expression assays to ensure measurable cellular responses given the lower sensitivity of these assays compared to MTT-based cell viability measurements. Future studies may optimize extract concentrations to enhance physiological relevance while maintaining assay sensitivity. Future studies may optimize extract concentrations to enhance physiological relevance while maintaining assay sensitivity.

Numerous studies have revealed that CBS shares similar effects with *C. bovis* in terms of basic properties, structural composition, and clinical efficacy (Xiang et al., 2019). Liu et al. demonstrated CBS's beneficial impact in rats with EE-induced intrahepatic cholestasis, increasing MRP2 and BCRP expressions (Liu et al., 2014; Xiang et al., 2019). Based on this information, we can conclude that among the bile acids and salts contained in *C. bovis*, chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) show cytotoxicity on OVCAR-3 (Horowitz et al., 2007). However, the exact mechanism of native *C. bovis* on these processes remains unclear. Among its implicate, *C. bovis* contains bilirubin, bile acids, amino acids, and various other substances (Yang and Wu, 2016). The bile acids CDCA and DCA acid have been reported to have a cytotoxic effect on OVCAR-3 cells by reducing apoptosis and protein kinase C (PKC) stimulation (Horowitz et al., 2007). Our study tested *C. bovis* extracts in various cell lines using three different solvents and revealed that the acetone extract exhibited strong cytotoxicity, especially over 90% activity against OVCAR-3 cells. These findings emphasise the efficacy of *C. bovis* against ovarian cancer, a disease with high incidence and mortality (Menon et al., 2021). In addition, the specificity of this study on the OVCAR-3 cell line contributed to the literature. Research by Horowitz et al. confirms that CDCA and DCA are among the bile acids that show cytotoxicity in an ovarian cancer cell line model (Horowitz et al., 2007). Studies have been limited to the cellular level, and various steroidal compounds and bile acids contained in *C. bovis* have been shown to have significant and highly cytotoxic effects on gynecologic cancers (Horowitz et al., 2007; Zhou, 2017). Further molecular investigations will yield potential for advancing cancer treatment strategies.

Breast cancer, a leading cause of cancer-related deaths in women, often treated with TEC (Taxotere, Epirubicin, and Cyclophosphamide) and CEF (Cyclophosphamide, Epirubicin, and Fluorouracil) regimens, benefits from adjunctive therapies like Xihuang Wan (XW), Ru Xiang (*olibanum*), Mo Yao (*Commiphora myrrha*), She Xiang (*Moschus*), Niu Huang'dan (*Calculus bovis*), and Xihuang Pill (XHP), historically used in traditional Chinese medicine (TCM) (Yang and Wu, 2016). A large part of the content of this drug consists of *C. bovis*. In addition, they demonstrated the immunomodulatory effects of XH in breast cancer patients in relation to the cytotoxic effects observed in MDA-MB-231 cells. Breast hyperplasia, particularly involving columnar epithelial cells, is associated with the earliest lesion that could be identified histologically in breast tissue that is linked to cancer progression. He mentioned that in addition to targeting tumors, XH has the capability to address benign lesions like breast hyperplasia and inhibit their progression into malignancies (Qiuju et al., 2015). In line with these observations, our study demonstrated that *C. bovis* extract exerted pronounced cytotoxic effects in the MDA-MB-231 cell line. These findings are consistent with the clarification provided by Qiuju et al. (2015) and further underscore the potential role of *C. bovis* in modulating cytotoxic responses in breast cancer cells. *C. bovis* has the potential to exert considerable chemotherapeutic effects in breast cancer treatment; however, further studies are warranted to elucidate the underlying molecular mechanisms.

Cancer development involves complex molecular processes influenced by oxidative stress-induced DNA damage, critical in carcinogenesis (Burçak and Andican, 2014). When evaluating the antioxidant capacity of *C. bovis*, it is thought that bilirubin, for example, the potential advantages of its antioxidant capabilities may confer some favorable effects (Qin, 2008). Unconjugated bilirubin is recognized as one of the most potent endogenous antioxidants. *C. bovis*, which is known to be rich in antioxidant components such as bilirubin, was evaluated using methanol extract in our study and a limited effect was observed in DPPH radical scavenging activity analysis. This suggests that the potential health benefits of *C. bovis* should be further investigated.

Apoptosis induction in cancer cells remains a pivotal strategy in cancer therapy, aiming to regulate proliferation and minimize healthy tissue damage. The mechanisms of apoptosis involve multiple molecular processes, and one of them, the intrinsic pathway, is governed by the induction or repression of *Bcl-2* family genes. In this process, *Bcl-2* promotes cell survival as an anti-apoptotic gene, while *Bax* plays an important role as a pro-apoptotic gene that initiates apoptosis (Akbaş et al., 2020; Coşkun and Özgür, 2011; Ou et al., 2021). Factors such as DNA damage, genotoxic stress and mutations in the *p53* gene cause triggering of the intrinsic pathway. *p53*, as a tumor suppressor gene, initiates apoptotic processes against genotoxic stress and becomes an important target for cancer therapy. These factors are induced as a response to cellular signals, and the expression of the gene is inhibited in most cancer cells (Bukholm and Nesland, 2000). This makes *p53* a suitable gene candidate for cancer therapy.

The results show that both *C. bovis* methanol extract and doxorubicin significantly downregulated *Bcl-2* gene expressions ($p < 0.0001$), suggesting that apoptotic processes may be suppressed or cells may turn to alternative signaling mechanisms. Furthermore, decreased *p21* and *p53* gene expression ($p < 0.0001$ and $p < 0.001$), cell cycle arrest, and tumor suppressor gene suppression suggest that apoptotic

processes may proceed by *p53* independent mechanisms. In contrast, no significant change in the expression of *Bax* was observed. These findings indicate that *C. bovis* methanol extract alters the expression levels of genes regulating apoptotic responses in OVCAR-3 cells, which may potentially impact cancer cells.

The simultaneous decrease in *Bcl-2* suggests that in addition to doxorubicin inducing apoptosis by causing DNA damage, cells may have turned to alternative mechanisms such as senescence (cellular aging) (Ou et al., 2021). The simultaneous decrease suggests that cells respond to the apoptotic process by a complex balancing mechanism and that other signaling pathways besides apoptosis may be involved. Furthermore, the cytotoxic effect of doxorubicin may lead to an overall suppression of cellular activity and, thus, reduced gene expression levels. This suggests that cells respond to the death signal with a complex balancing mechanism and that different signaling pathways besides apoptosis may be involved. For the different treatment groups, tabled results based on the identified *Bax/Bcl-2* ratios reveal the effects on apoptosis. The uninhibited group (0.741) has a high ratio of *Bax/Bcl-2* and is more likely to trigger apoptosis. Doxorubicin treatment (0.343) and *C. bovis* treatment (0.41) prevented apoptosis due to the reduction of the *Bax/Bcl-2* ratio, offering protection to the cell from apoptosis. The results indicate that both the Doxorubicin and *C. bovis* treatments were anti-apoptotic in cancer cells, promoting their survival, while also hindering the initiation of apoptosis. Sensitivity to treatment is positive, suggesting that cancer cells can be more readily killed and treatment can probably stimulate apoptosis. In contrast, the untreated group is more susceptible to undergo apoptosis or has a higher incidence to initiate apoptosis than the treated group. Thus, treatment insensitivity means an impaired apoptosis, thus maybe limiting the efficacy of the treatment. These findings indicate that *C. bovis* extracts and doxorubicin may affect cellular responses through various mechanisms.

Animal preparations such as *C. bovis* are important sources of biologically active compounds because of their important bioactive properties. The findings of this study confirmed that acetone ethanol and methanol extracts of *C. bovis* showed anticancer activity (OVCAR-3 and MDA-MB-231). In addition, there is not enough data on the anticancer, antioxidant and apoptosis initiation mechanisms of *C. bovis* and its extracts. The current investigation demonstrated that the extracts suppressed the proliferation of OVCAR-3 and MDA-MB-231 cancer cells by triggering the apoptotic pathway. The findings of this research could a different perspective on the evaluation of the therapeutic properties of extracts of natural *C. bovis* in scientific studies. Further research is needed to deepen your comprehension of the molecular mechanisms that underlie the apoptosis-induced effect and anti-proliferative activity of *C. bovis*. We think that *C. bovis* can be a good chemotherapeutic agent against cancer researches due to its anticancer effects thanks to its rich compounds and that more research on this subject should be conducted. The data findings gathered from the investigation showed that *C. bovis* extracts have inhibitory potential against human cancer cells at low concentrations, especially in human ovarian and breast cancer cells. The data obtained from the investigation; It provides preliminary data for medical, pharmaceutical and pharmacological studies that may be beneficial in cancer treatment with its anticancer and cytotoxic effects and sheds light on future research in this field.

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