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RESEARCH PAPER

Cytotoxic activity of methanolic and ethanolic extract of *Aquilaria agallocha Roxb.* heartwood against healthy fibroblast and breast cancer cells

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Abstract

Aquilaria agallocha Roxb. has traditionally been used to treat various medical conditions, including inflammation, fever, and cardioprotection. However, limited studies have explored its specific effects on cancer cells while distinguishing its impact on normal cells. This study aimed to evaluate the effects of *A. agallocha* heartwood extracts on both normal and cancer cells. To achieve this, mouse L929 (normal), 67NR, and 4T1 (cancer) cells were treated with methanol and ethanol extracts of *A. agallocha* heartwood to assess cytotoxicity, apoptosis, and oxidative stress. Flow cytometry analysis revealed that both extracts induced apoptosis and oxidative stress in all tested cell lines. However, the extracts exhibited a greater cytotoxic effect on normal L929 cells, potentially due to their bioactive components exerting a stronger pro-oxidant effect. These findings suggest that *A. agallocha* extracts may differentially affect normal and cancer cells, highlighting the need for further investigation into their selective cytotoxicity.

Introduction

Natural bioactive compounds, including terpenoids, phenylpropanoids, alkaloids, polyphenols, carotenoids, phytosterols, tocopherols, tocotrienols, organosulfur compounds, peptides, and fibers, have demonstrated great potential in drug development for various diseases (Abo-Elghiet et al., 2020). These compounds, derived from natural sources such as plants, offer diverse therapeutic properties that can be harnessed to treat various ailments (Prasad et al., 2020). Such natural compounds (NCs) provide a rich source of chemical diversity, making them valuable for discovering and developing new medications. Historically, natural compounds have been crucial in drug development, particularly in the treatment of cancer and infectious diseases, as well as in other medical areas such as cardiovascular diseases and multiple sclerosis (Atanasov et al., 2021; Harvey et al., 2015; Newman et al., 2016; Tintore et al., 2019). NCs generally have a higher molecular mass and a greater presence of sp3-hybridized carbon and oxygen atoms. Furthermore, they tend to have fewer nitrogen and halogen atoms and more hydrogen bond acceptors and donors. They also have lower calculated octanol-water partition coefficients (cLogP values), indicating that they are more hydrophilic (Clardy & Walsh, 2004; Feher & Schmidt, 2003). Moreover, they are usually more molecularly rigid than synthetic compound libraries, and this higher rigidity can be beneficial in drug discovery for

addressing protein-protein interactions (Doak et al., 2014; Lawson et al., 2018). Additionally, natural bioactive compounds have been thoroughly investigated for their potential role in cancer therapy. Numerous compounds demonstrate anticancer effects by focusing on essential cellular pathways related to tumor progression, apoptosis, angiogenesis, and metastasis. Polyphenols, such as curcumin and resveratrol, have shown the ability to induce apoptosis and inhibit the proliferation of cancer cells. Besides, terpenoids such as paclitaxel and vinca alkaloids like vinblastine and vincristine are widely used in chemotherapy due to their strong cytotoxic effects on cancer cells. NCs are essential for the development of innovative anticancer therapies due to their diverse mechanisms of action and significantly lower toxicity compared to synthetic drugs (Limam et al., 2024; Sytar and Smetanska, 2022).

Aquilaria agallocha Roxb. (*A. agallocha*) is a well-known herbal plant from the Thymelaeaceae family. In addition to being used in industrial products such as gum, flavouring or perfume, it is also used as a folk medicine in treating diseases such as fever, diarrhea, cardioprotection, and anti-inflammation (Alam et al., 2021; Nguyen et al., 2023). Furthermore, herbal compounds such as polyphenols, flavanols, alkaloids, and terpenoids are known for their anti-cancer properties. Ethanol is a good solvent for extracting polyphenols, and alkaloids are safe for human consumption. Methanol is generally more efficient in extracting lower molecular weight polyphenols and effective for extracting higher molecular weight flavanols. Furthermore, these polyphenols, flavanols, alkaloids, and terpenoids are strong inducers of reactive oxygen species (ROS) in cells. A moderate amount of ROS is crucial for cellular signaling, which controls cell proliferation and survival (Trachootham et al., 2009). However, high levels of ROS can harm cell structures like lipids, proteins, and DNA, leading to an imbalance in cellular redox conditions and disrupting homeostasis (Samson and Nelson, 2000). Many ROS-related anticancer therapy studies have been developed based on the imbalance of free radicals and antioxidants by accelerating accumulative ROS in cancer cells (Chakrabarti et al., 2015; Li et al., 2019; Perillo et al., 2020). The overproduction of ROS plays a critical role in cell signaling and regulates the main pathways of apoptosis mediated by mitochondria, death receptors, and the ER (Li et al., 2018). Furthermore, apoptosis is typically categorized into extrinsic and intrinsic pathways involving the mitochondria. The extrinsic pathway begins when death ligands bind to their receptors, forming and stimulating caspase-8 and activating effector caspases like caspase-3 (Kashyap et al., 2021). The intrinsic pathway, in contrast, starts with cytochrome c being released from the mitochondria into the cytosol as a result of increased mitochondrial permeability. The cytosolic cytochrome c release activates effector caspases via apoptosome formation, consisting of Apaf-1, cytochrome c, and caspase-9. This pathway is specifically regulated by members of Bcl-2 family proteins, like Bax (Carneiro & El-Deiry, 2020).

However, many plant extracts contain compounds that may act on both healthy and cancerous cells. These compounds can damage both cell types by interfering with the cells' general biochemical processes (Jaramillo-Rangel et al., 2020). However, these targets can be found not only in cancer cells but also in normal healthy cells. This may furthermore cause damage to normal healthy cells. While normal healthy cells may be unable to detoxify some toxic compounds, cancer cells can detoxify these compounds more effectively through active antioxidant mechanisms (Kundishora et al., 2020). These factors may help explain why plant extracts can sometimes harm normal healthy cells more than cancer cells. Research on herbal treatments and extracts aims to develop more targeted and selective compounds to minimize these effects (Raheel et al., 2017). However, studies on heartwood from *A. agallocha* anticancer activity remain sparse. The results of these studies are controversial, and some did not even use normal healthy cells to compare the effect of *A. agallocha* extract (Abbas et al., 2019; Dahham et al., 2014; Nahar et al., 2023).

Although the effects of extracts from the A. agallocha plant on cancer cells have been evaluated in some studies, unfortunately, their effects on normal healthy cells have not been studied. In order to precisely examine whether a plant extract has an anti-cancer effect, the effects of the extract on normal healthy cells must also be understood. Therefore, to evaluate the cytotoxic and especially apoptotic effects of *A. agallocha* heartwood extract, the L929 normal healthy mouse fibroblast cells, 67NR mouse primary breast cancer cells, and 4T1 mouse metastatic breast cancer cells were subjected to A. agallocha methanol and ethanol extract. Overall, this study was performed to determine the level of cellular toxicity on *A. agallocha* heartwood methanol and ethanol extract-treated normal healthy fibroblast and breast cancer cells.

Materials and Methods

Preparation and application of *A. agallocha* heartwood sample extract

Ten grams of *A. agallocha* heartwood samples were ground into a fine powder using a laboratory sample preparation mill, and then an equal amount of 5g of samples was dissolved in 5 mL of pure methanol and ethanol. The dissolved methanol and ethanol samples were incubated at room temperature on a magnetic stirrer for 24 h. After the incubation period, the samples were filtered through a 40 µm nylon filter to eliminate heartwood residues, and then passed through a 0.22 µm polyethersulfone (PES) filter to prevent bacterial contamination before being poured into the 15 mL conical tubes. The cap of the tube containing the 5 mL samples of methanol and ethanol

was loosened, and the filtrated samples were incubated to dry by vacuum-evaporation (37°C, 40 bar) for 48 h to obtain the crude extracts. After 48 h incubation, the crude extracts of methanol and ethanol samples were weighed, and it was found that 1400 mg of crude methanol and 1200 mg of crude ethanol extracts were obtained. Extraction yield percentage is obtained according to the final dried crude extract weight in mg obtained relative to the initial weight of 5000 mg heartwood dissolved in 5 mL solvent. The following Formula was used to calculate the extraction yield percentage: % Yield= (Final weight (mg)/Initial weight (mg)) X 100. As a result, the final extraction yield percentage was 28% with methanol extraction and 24% with ethanol extraction from *A. agallocha* heartwood samples. The obtained crude extracts were then dissolved in 1 mL of Dimethyl sulfoxide (DMSO), and as a result, 1400 mg/mL and 1200 mg/mL methanol and ethanol extract stock solutions were obtained. After completing all the procedures, the resulting extract was kept at -20°C for additional processing. Concentrations from a prepared stock solution of methanol and ethanol extracts (200, 100, 80, 60, 40, and 20 µg/mL) were applied concurrently to normal healthy cells and cancer cell lines for treatment (Batir et al., 2023).

Culturing conditions of cell lines

Mouse fibroblast cell line L929, mouse breast primary cancer cell line 67NR, and mouse breast metastatic cancer line were purchased from American Type Culture Collection (ATCC). L929, 67NR, and 4T1 cells were cultured in a medium containing RPMI 1640, 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin, and 1% L-Glutamine at 37°C in a 5% CO_2 incubator. When the cells reached confluence, they were subcultured to ensure growth. Cells were cultured in 24-well plates with 60000 cells in each well, followed by incubation with the *A. agallocha* extracts at related concentrations for 24 and 48 h. All experiments were performed in duplicate for each extract (Batir et al., 2023).

In vitro cytotoxicity analysis

Cytotoxicity analysis of L929, 67NR, and 4T1 cells was carried out using flow cytometry. The extract-treated cells were washed with Dulbecco's phosphate-buffered saline 1X (DPBS) and treated with trypsin. After trypsinization, the cells were centrifuged, suspended, and diluted in a complete medium. After dilution, the total cell concentrations and viability were determined using the Muse Count & Viability reagent (Merck Millipore) and the automated Muse® Cell Analyser (Merck Millipore).

Apoptosis analysis

The apoptotic effects in L929, 67NR, and 4T1 cells were evaluated. These cells were grown in 24-well plates and then treated with *A. agallocha* methanol and ethanol extracts for 24 and 48 h at their half-maximal inhibitory concentration (IC_{50}) values. Following the post-incubation period, the cells were labeled using the Muse Annexin V and Dead Cell kit according to the manufacturer's guidelines. The Muse flow cytometry instrument was used to analyse the cells and calculate the rate of apoptosis (Batır et al., 2019).

Gene expression analysis of anti-apoptotic and pro-apoptotic target genes

RNA was extracted from L929, 67NR, and 4T1 cells cultured in 24-well plates and exposed to *A. agallocha* methanol and ethanol extracts at IC_{50} concentrations for 24 and 48 h using the Quick-RNA Miniprep Kit (ZymoResearch) according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription kit was used to conduct cDNA synthesis, following the protocol provided by the manufacturer. The process took place in the SensoQuest thermal cycler using isolated total RNAs as samples. After this, to perform gene expression analysis of target genes on cDNAs Bcl-2 (BCL2F1 5'-GGGGTCATGTGTGTGGAGAG-3', BCL2R1 5'-CATCCCAGCCTCCGTTATCC-3'), Bcl-xL (BCLxLF1 5'-AGTAAACTGGGGTCGCATCG-3', BCLxLR1 5'-GGCCATCCAACTTGCAATCC-3') Caspase-3 (CASP3F15'-ACATGGGAGCAAGTCAGTGG-3', CASP3R1 5'-CCGTACCAGAGCGAGATGAC-3'), Bax (BAXF1 5'-GATCATGAAGACAGGGGCCT-3', BAXR1 5'-AGACACTCGCTCAGCTTCTT-3'), Caspase-8 (CASP8F1 5'-TCCTAGACTGCAACCGAGAG-3', CASP8R1 5'-TCCAACTCGCTCACTTCTTCT-3'), Caspase-9 (CASP9F1 5'-GGACCGTGACAAACTTGAGC-3', CASP9R1 5'-TCTCCATCAAAGCCGTGACC-3'), and Cytochrome C (COX1F1 5'-CCTCTTCGTCTGATCCGTCC-3', COX1R1 5'-TGAGGGTTGCGGTCTGTTAGT-3') primer pairs were designed. Analysis of target gene expression was performed by using a 25 µL total reaction mixture. This mixture consisted of 2 µL cDNA, 1.25 µL of EvaGreen, 0.3 µL of 10 pmol Primer Forward, 0.3 µL of 10 pmol Primary Reverse, 12.5 µL of 2X Hot-start master mix, and 8.65 µL of ddH_2O. The analysis was carried out in a Rotor-Gene Real-Time PCR (Qiagen, USA) device. The housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) was used for the normalization of target genes, as it exhibited the most stable expression values. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the normalized expression values of the target genes.

Oxidative stress analysis

L929, 67NR, and 4T1 cells were seeded in a 24-well plate (60000 cells/well) and incubated for 16-18 h. After incubation, these cells were treated with *A. agallocha* methanol and ethanol extracts for 24 and 48 h at their IC_{50} values. After the post-incubation period, the cells were stained using the Muse Oxidative Stress Kit as per the guidelines provided by the manufacturer. The Muse flow cytometry instrument was used to analyse the cells and determine the rate of reactive oxygen species (ROS) positive cells.

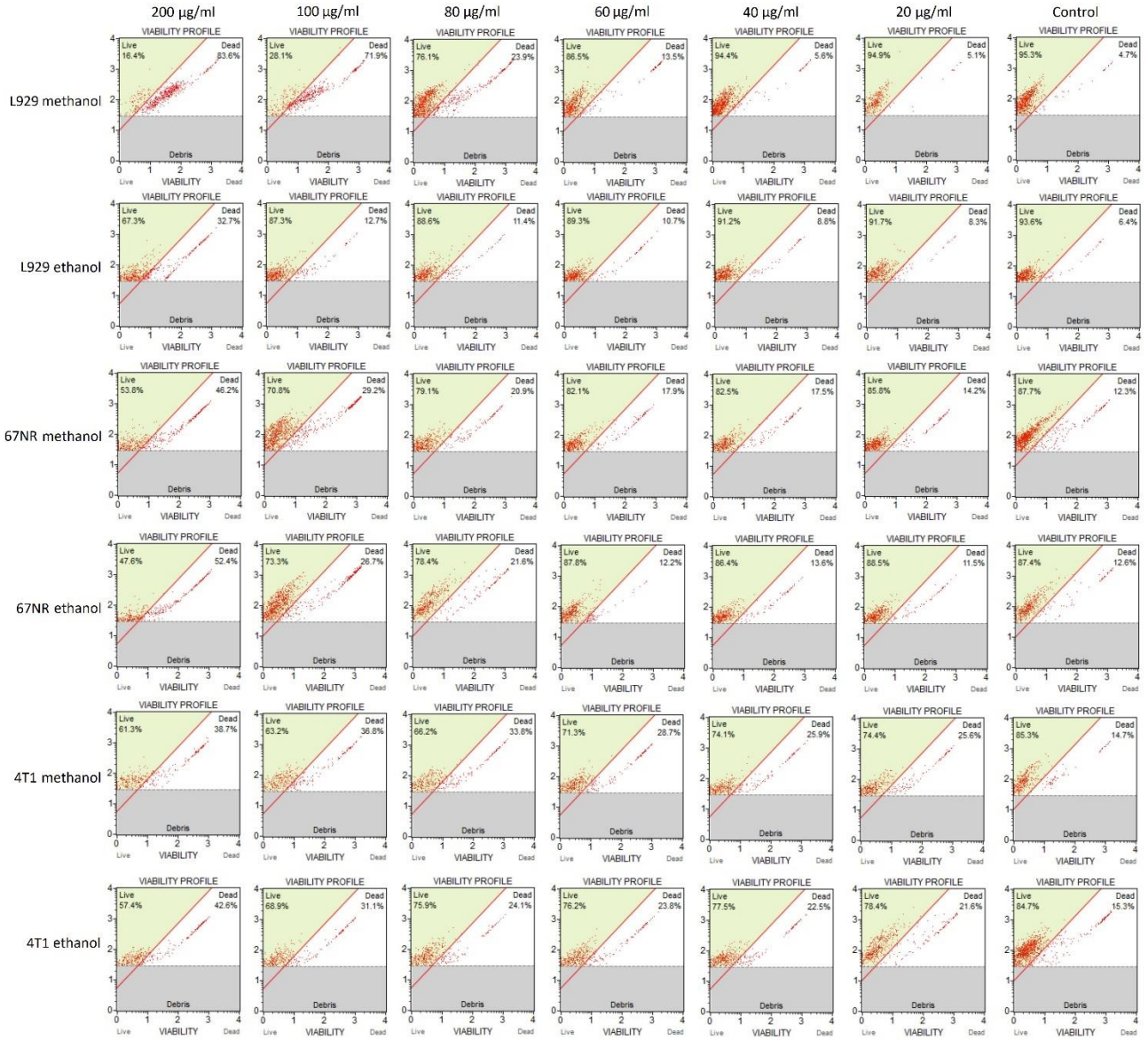

Figure 1. Flow cytometry viability values of L929, 67NR and 4T1 cells treated with 200, 100, 80, 60, 40 and 20 µg/mL *A. agallocha* methanol and ethanol extract for 24 h.

Statistical analysis

The differences observed between the groups were analysed using SPSS (Statistical Package for the Social Sciences, Windows 23.0). The data obtained underwent testing through One-way ANOVA and a Tukey post hoc test. Any p-values less than 0.05 were considered significant. The findings were then expressed as the mean ± standard deviation (SD). Furthermore, IC_{50} values after the flow cytometry viability assay were calculated with GraphPad Prism 9.

Results

Evaluation of cytotoxicity of *A. agallocha* methanol and ethanol extract treatment on L929, 67NR and 4T1 cells

The methanol and ethanol extracts of *A. agallocha* were applied to L929, 67NR, and 4T1 cells at 200, 100, 80, 60, 40, and 20 µg/mL doses for 24 and 48 h. Subsequently, the cell viability was measured using flow cytometry. It was found that the viability of L929, 67NR, and 4T1 cells in groups treated with methanol and ethanol extracts decreased in a dose-dependent manner after 24 h of treatment (Figure 1). Furthermore, it was found that the viability of L929, 67NR, and 4T1 cells in groups treated with methanol and ethanol extracts decreased dose-dependent after 48 h of treatment, the same as the 24 h of the extract-treated groups (Figure 2). The microscopic analysis results of the methanol and ethanol extract treatment are also shown in Figure 3.

According to the viability values shown in Figure 4, the IC_{50} values of L929, 67NR, and 4T1 cells, which were treated with methanol extract for 24 h, were found to be 59.53 µg/mL, 105.45 µg/mL, and 109.16 µg/mL, respectively. Furthermore, the IC_{50} values of L929, 67NR, and 4T1 cells, treated with ethanol extract for 24 h, were found to be 101.06 µg/mL, 138.6 µg/mL, and 146.4 µg/mL, respectively. On the other hand, the IC_{50} values of L929, 67NR, and 4T1 cells, which were treated with methanol extract for 48 h, were found to be 67.14 µg/mL, 102.2 µg/mL, and 110.23 µg/mL, respectively.

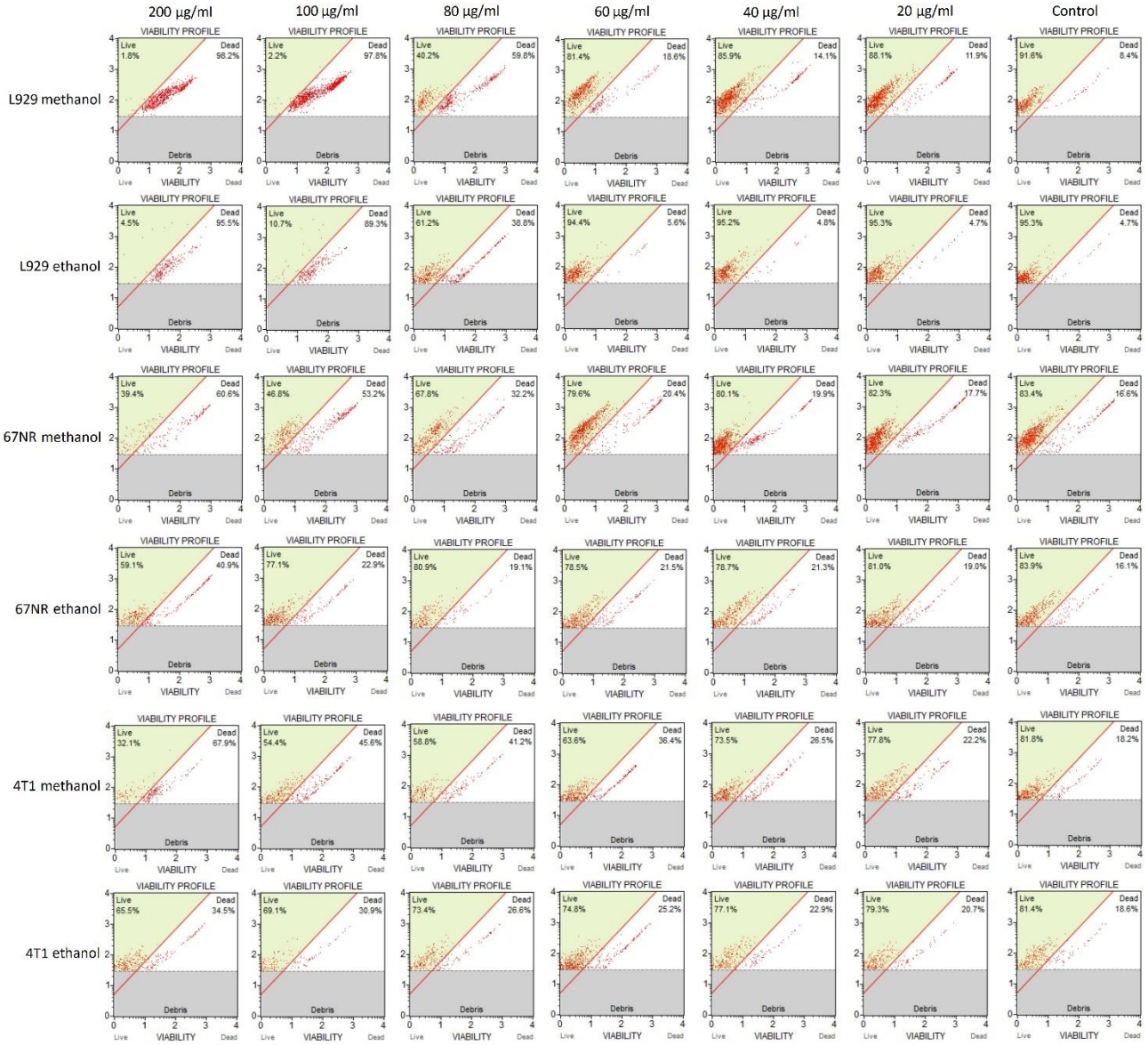

Figure 2. Flow cytometry viability values of L929, 67NR and 4T1 cells treated with 200, 100, 80, 60, 40 and 20 µg/mL *A. agallocha* methanol and ethanol extract for 48 h.

Additionally, the IC_{50} values of L929, 67NR, and 4T1 cells, treated with ethanol extract for 48 h, were found to be 86.54 µg/mL, 131.56 µg/mL, and 137.13 µg/mL, respectively.

Effect of *A. agallocha* methanol and ethanol extracts on apoptosis induction of L929, 67NR and 4T1 cells

Measuring the apoptosis induction rate of *A. agallocha* methanol and ethanol extract was done using the flow cytometry method. This method allows for the determination of live (AnnV-/PI-), early apoptotic (AnnV+/PI-), late apoptotic (AnnV+/PI+), and necrotic (AnnV-/PI+) cells. IC_{50} values derived from cell viability data were administered to L929, 67NR, and 4T1 cells for 24 and 48 h. The Muse Annexin V and Dead Cell kit were used to assess the apoptotic process in the cells. It was determined that the viability rate decreased, and apoptotic cell number increased significantly for 24 and 48 h in L929 cells treated with methanol and ethanol IC_{50} concentrations, according to the 67NR and 4T1 cancer cells ($p<0.0001$) (Figure 5). After 24 h of methanol extract treatment, Figure 5 displays the percentage of apoptotic (early and late apoptosis) cells for L929, 67NR, and 4T1 cells as 41.4±0.52%, 33.3±0.55%, and 33.5±0.17%, respectively. After 24 h of ethanol extract treatment, the percentage of apoptotic (early and late apoptosis) cells observed for L929, 67NR, and 4T1 cells was 42.7±0.56%, 36.8±0.26%, and 31.7±0.44%, respectively. Additionally, after 48 h of methanol extract treatment, the percentage of apoptotic (early and late apoptosis) cells was observed to be 44.6±0.47%, 37.1±0.37%, and 39.2±0.73% for L929, 67NR, and 4T1 cells, respectively. Following 48 h of ethanol extract treatment, the percentage of apoptotic (early and late apoptosis) cells was measured at 40.3±0.9%, 32.2±0.6%, and 30.3±1.21% for L929, 67NR, and 4T1 cells, respectively.

***A. agallocha* induced apoptosis by controlling apoptotic gene expression**

RT-qPCR analyses of anti-apoptotic Bcl-2, Bcl-xL, and pro-apoptotic Caspase-8, Bax, and Cytochrome C

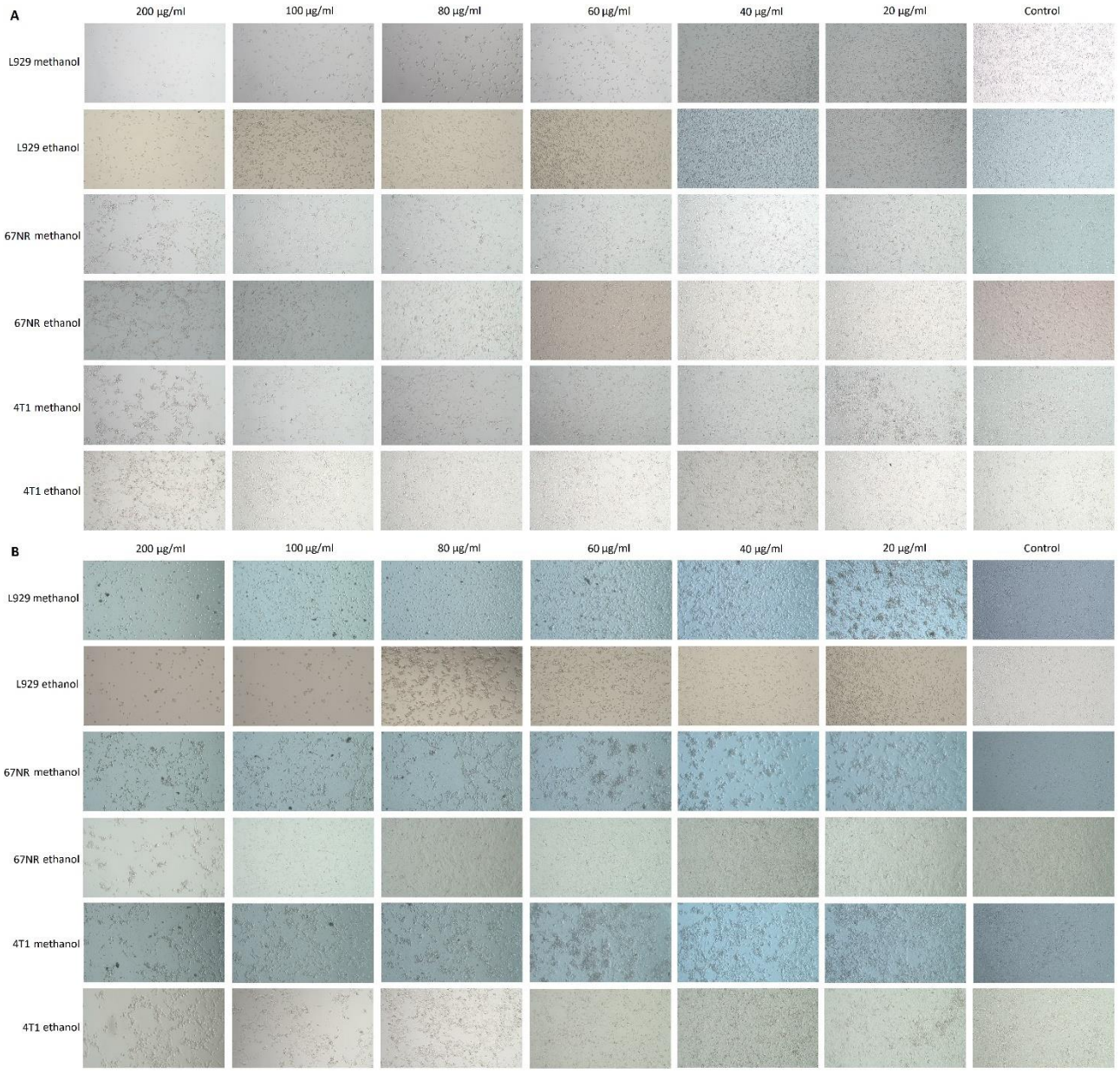

Figure 3. Microscope images of L929, 67NR and 4T1 cells treated with 200, 100, 80, 60, 40 and 20 µg/mL *A. agallocha* methanol and ethanol extract for 24 **A**) and 48 **B**) h (x20 magnification).x

genes were performed on cells treated with *A. agallocha* methanol and ethanol extract at IC_{50} concentrations was applied for 24 and 48 h. As seen in Figure 6, *A. agallocha* methanol and ethanol extract increased the mRNA expression level of pro-apoptotic Caspase-3, Caspase-8, Caspase-9, Bax, and Cytochrome C genes in L929, 67NR and 4T1 cells, while decreasing the anti-apoptotic Bcl-2, Bcl-xL mRNA expression level. However, it was determined that the change in Caspase-3, Caspase-8, Caspase-9, and Cytochrome C gene expression levels was higher in L929 cells compared to 67NR and 4T1 cells ($p<0.0001$) for 24 and 48 h of methanol and ethanol extract treatment.

***A. agallocha* methanol and ethanol extract increased the intracellular oxidative stress in L929, 67NR and 4T1 cells**

L929, 67NR, and 4T1 cells were treated with IC_{50} concentrations of the *A. agallocha* methanol and ethanol extract for 24 and 48 h to measure the ROS-positive cell population using a Muse cell analyser. The ROS-positive cell population was increased according to the treatment of the IC_{50} concentrations of the *A. agallocha* methanol and ethanol extract for 24 (Figure 7A) and 48 (Figure 7B) h. However, it was found that the increased ROS-positive L929 cell number was higher rather than the ROS-positive 67NR and 4T1 cell number after the 24 and 48 h of *A. agallocha* methanol and ethanol extract treatment of the cells ($p<0.0001$).

Discussion

Medicinal plants have been used as traditional treatments for various human diseases for thousands of years (Sidhu et al., 2020). Our study explored the potential of *A. agallocha* as a bioactive substance derived from natural sources, examining its pharmacological effectiveness in anti-cancer properties

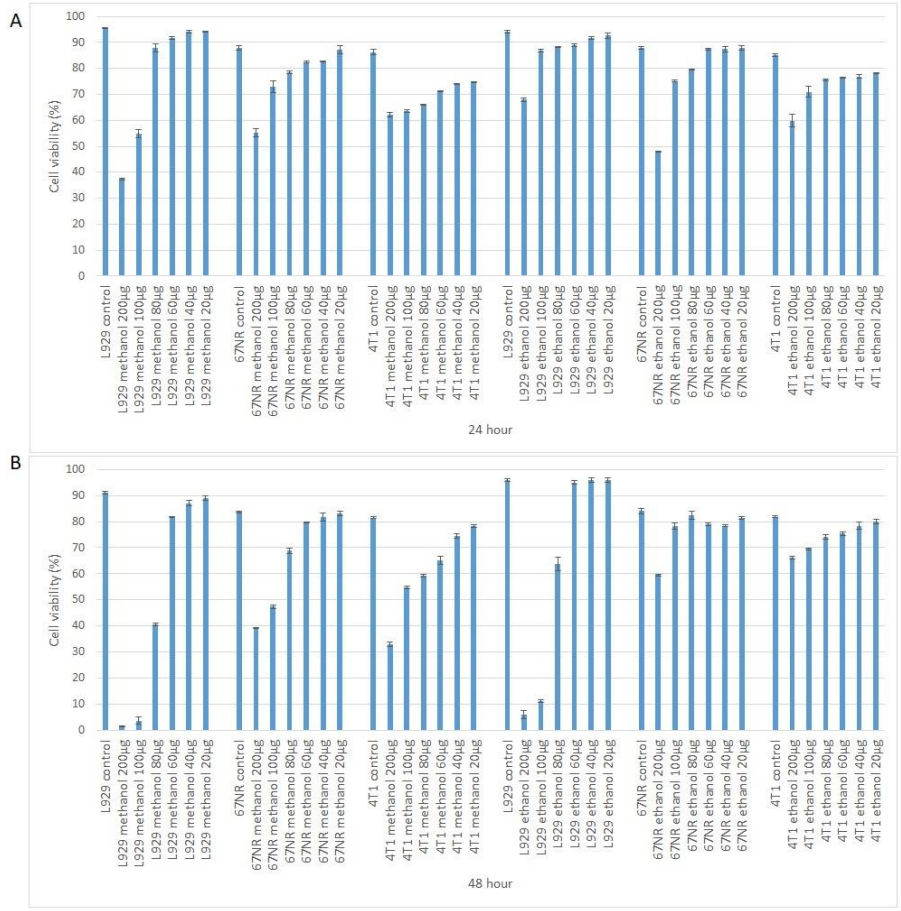

Figure 4. The viability values of L929, 67NR and 4T1 cells treated with 200, 100, 80, 60, 40 and 20 µg/mL *A. agallocha* methanol and ethanol extract for 24 **A)** and 48 **B)** h.

through in vitro experiments. In order to evaluate the toxicity of methanol and ethanol extracts obtained from the heartwood of *A. agallocha*, these extracts were tested for cytotoxic effects against L929, 67NR, and 4T1 cells.

In the cell viability analysis, 24- and 48-h treatment of *A. agallocha* methanol and ethanol extracts resulted in higher IC_{50} concentrations in 67NR and 4T1 cancer cells. While it was determined to be effective on healthy L929 fibroblast cells, it was observed to be effective at lower IC_{50} concentrations. Nahar et al. (2023) obtained *A. agallocha* extract with 60% ethanol solution from the wood part of the plant. Then, they used this extract to treat healthy keratinocyte HaCaT obtained from adult human skin, macrophage RAW 264.7 obtained from tumour tissue of male mice, and lung adenocarcinoma human alveolar basal epithelium A549 cells. As a result of this study, it was observed that 400 µg/mL concentration of *A. agallocha* extract application caused a 60% cell death ratio in healthy keratinocyte cells. In comparison, 1000 µg/mL concentration caused only a 20% cell death ratio in RAW 264.7 cells, and 1000 µg/mL concentration caused only a 40% cell death ratio in A549 cells. Similarly, as in our study, Nahar et al. (2023) showed that while *A. agallocha* extract had a higher effect on normal healthy cells at low concentrations, it had a limited effect on cancer cells at higher concentrations. In another study, the cytotoxic effect of the extract obtained from the root bark of the *Aquilaria crassna* plant, which is in the same genus as *A. agallocha* and whose aromatic wood region is used for various purposes, was tested on healthy and cancer cells (Dahham et al., 2014). In this study, the IC_{50} values of the ethanol extract obtained from the *A. crassna* were treated to healthy human endothelial HUVEC (IC_{50}=48 µg/mL), human pancreatic cancer PANC-1 (IC_{50}=72 µg/mL), human prostate cancer PC-3 (IC_{50}=119 µg/mL), human colon cancer HCT-116 (IC_{50}=30 µg/mL) and human breast cancer MCF-7 (IC_{50}=140 µg/mL) cells. As a result, they found that *A. crassna* ethanol extract showed more toxic effects on healthy cells rather than cancer cells, except for HCT-116 colon cancer cells. In their studies, the authors stated that *Aquilaria* spp. (Thymelaeaceae) extracts had a cytotoxic effect on cancer cells, but this effect was seen to be greater on normal healthy cells. For a substance to have anti-cancer activity, the toxic activity must be specific to cancer cells, as plant extracts may have limited effect on normal healthy cells (Solowey et al., 2014). Apart from these studies, it was found that the ethanol extract from the leaves of the *Aquilaria malaccensis*, which is in the same genus as *A. agallocha*, showed toxicity at higher concentrations on healthy mouse fibroblast NIH/3T3 cells and lower concentrations on human breast cancer MCF-7 cells (Aziz et al., 2023).

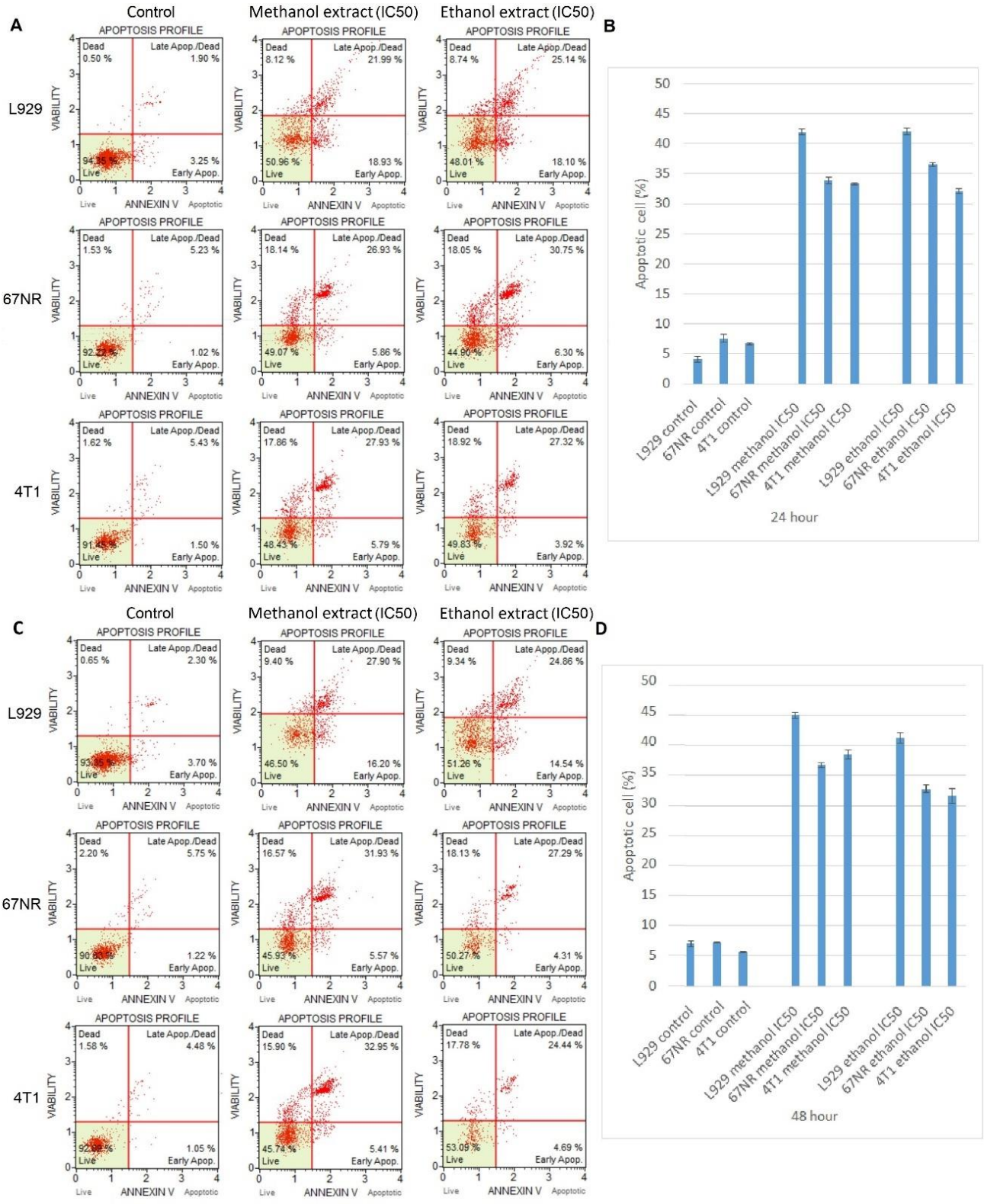

Figure 5. Apoptosis graphs of *A.agallocha* methanol and ethanol extract treated L929, 67NR and 4T1 cells. Apoptosis flow cytometry graphs of methanol and ethanol extract treated cells for 24 h **A**), apoptosis bar graph results of methanol and ethanol extract treated cells for 24 h **B**), apoptosis flow cytometry graphs of methanol and ethanol extract treated cells for 48 h **C**), and apoptosis bar graph results of methanol and ethanol extract treated cells for 48 h **D**).

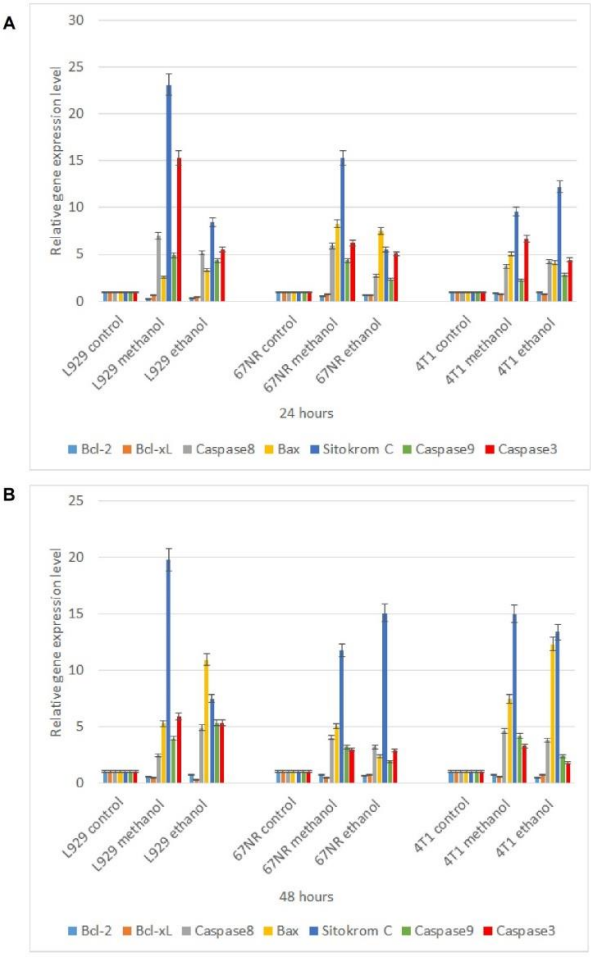

Figure 6. RT-qPCR analysis results of anti-apoptotic and pro-apoptotic gene expression levels of L929, 67NR and 4T1 cells treated with *A. agallocha* methanol and ethanol extract at IC_{50} concentrations for 24 h **A**) and 48 **B**) h.

It is crucial to identify the various types of programmed cell death (PCD) in the cell group treated with the agent (Batir et al., 2023). PCD encompasses death mechanisms like apoptosis, autophagy, necroptosis, and pyroptosis (Ketelut-Carneiro & Fitzgerald, 2022). Hence, it is essential to determine the various types of cell death mechanisms in the cells treated with the agent. In processes involving living organisms, apoptosis is the most common mechanism of cell death (Bertheloot et al., 2021). Therefore, this study investigated whether cell deaths in cell viability analysis occurred through apoptosis. For this reason, cells to which *A. agallocha* extract was applied were examined by flow cytometry analysis. As a result of the analysis, it was determined that these deaths in the cells in both 24 and 48-h applications were caused by apoptosis. Additionally, it was observed that the total apoptotic cell rate was higher in healthy L929 cells, to which extract was applied with a lower concentration IC_{50} value than in 67NR and 4T1 breast cancer cells, both at the 24th and 48th h. On the other hand, studies carried out by Nahar et al. (2023) and Dahham et al. (2014) did not perform apoptosis analyses after treating the cells with Aqualaria sp. extract. Apart from them, only Aziz et al. (2023) examined the apoptotic process by AnnexinV/PI analysis on the Aquilaria spp. extract treated cells. The results obtained by Aziz et al. (2023) determined that *Aquilaria* spp. extract triggered the apoptotic process in cells.

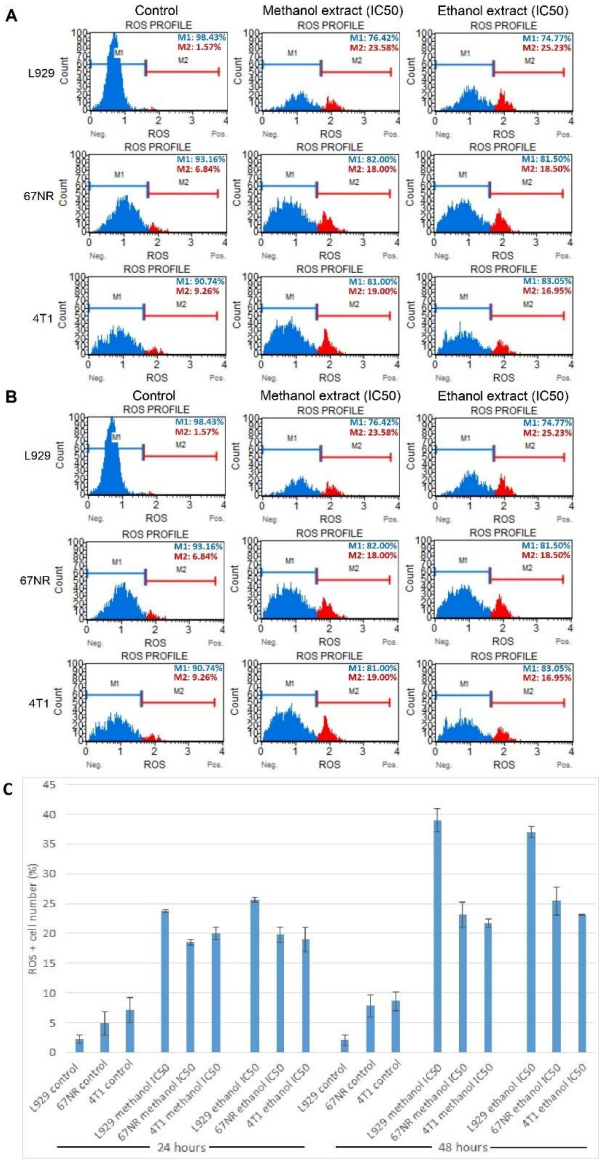

Figure 7. Effects of *A. agallocha* methanol and ethanol extract on ROS generation. L929, 67NR, and 4T1 cells treated with IC_{50} concentrations of *A. agallocha* methanol and ethanol extract and then analysed for the ROS-/+ cell population values at 24 h **A**) and 48 h **B**). M1 represents ROS- cell population and M2 represents the ROS+ cell population. ROS bar graph results of L929, 67NR and 4T1 cells treated with *A. agallocha* methanol and ethanol extract at IC_{50} concentrations for 24 and 48 h **C**).

The ability of a substance to trigger cell death in cancer cells shows promise for its use as an anti-cancer treatment. Several plant-derived anti-cancer compounds that induce apoptosis have been approved for clinical trials. The anti-cancer treatment kills cancer cells and inhibits their growth. Apoptosis induction is a more profound mechanism of cell death triggered by naturally occurring anti-cancer agents. Evading apoptosis is the major challenge in killing cancer cells, a mechanism mainly regulated intrinsically and

extrinsically (Chaudhry et al., 2022). Therefore, we also analysed the potential of *A. agallocha* extracts to induce cell death by studying the mRNA expression of genes related to apoptotic cell death. Additionally, identifying the active genes underlying the death mechanism requires understanding which gene signaling pathways are involved (Yau et al., 2015). During the apoptotic process, many pro- and anti-apoptotic genes belonging to the BCL-2 (Bcl-2, Bcl-xL, Bax, Bak, etc.) and caspase (caspases-3, -8 and -9, etc.) family play a role. BCL-2 and members of the caspase family, one of the pathways leading to PCD, are activated in response to stress conditions such as DNA damage or cytokine deficiency (Luna-López et al., 2010). In this case, down-regulation of Bcl-2 and Bcl-xL gene expression, up-regulation of caspase-3, caspase-8, caspase-9, and Bax gene expression, and release of cytochrome C into the cytoplasm are observed (Rajabi et al., 2021). In this study, it was determined that Bcl-2 and Bcl-xL gene expressions were significantly downregulated, and Bax, caspase-3, -8, -9, and cytochrome C genes were significantly upregulated compared to controls in L929, 67NR, and 4T1 cells treated with *A. agallocha* extracts. In the study of Nahar et al. (2023), similar to our study results, Bcl-2 gene expression was downregulated, and Bax, caspase-3, and -9 gene expression levels were upregulated in A549 cells which were treated with *A. agallocha* extracts. Unfortunately, Nahar et al. (2023), since no gene expression studies were performed on normal healthy cells, it could not be determined whether the main effect of *A. agallocha* extract had more effect on mRNA expression levels in healthy or cancer cells. On the other hand, in our study, it was determined that there were higher levels of the changes caused by *A. agallocha* extract in Bcl-2 and Bcl-xL, caspase-3, caspase-8, caspase-9, and cytochrome C gene expression levels in healthy L929 cells compared to 67NR and 4T1 cancer cells, just as in cytotoxicity and apoptosis analyses. In addition, it has been determined that *A. agallocha* extract causes both healthy and cancer cells to undergo apoptosis through internal pathways by increasing caspase-9 expression and external pathways by increasing caspase-8 expression.

ROS plays a role in various cellular activities, and maintaining ROS at low to moderate levels appears necessary for cell survival (Lee et al., 2021). Nevertheless, an overabundance of ROS leads to cell death by triggering DNA damage-related apoptotic pathways (Guo et al., 2023; Li et al., 2017). For this reason, oxidative stress, an indicator of ROS production in cells, was evaluated to determine whether cell death was due to DNA damage. This study revealed that the *A. agallocha* extract treatment on cells increased ROS-positive cell number and ROS-induced apoptotic effect. These findings imply that the rise in ROS production is linked to *A. agallocha*-triggered apoptosis.

Conclusion

Overall, this study determined that *A. agallocha* extract, within a defined concentration range, caused the death of mouse L929 fibroblast cells, mouse 67NR primary breast cancer cells, and mouse 4T1 metastatic breast cancer cells through the apoptotic process. Furthermore, *A. agallocha* extract induced the activation of multi-caspases, which accompanied the increase in ROS generation. However, it was determined that *A. agallocha* extract had a greater effect on healthy L929 cells when cytotoxicity, apoptosis, RT-qPCR, and oxidative stress analyses were examined. This study evaluated the therapeutic effect of extracts obtained from the heartwood part of the *A. agallocha* plant on cancer cells, which is one of the limitations of our study. In the next study, advanced analyses of extracts obtained from the leaves of the *A. agallocha* plant on cancer cells will be performed. Additionally, according to the results of this study, it is unsafe to use extracts obtained from the heartwood parts of the *A. agallocha* or grind the heartwood parts in powder form for cancer treatment. It has been seen that treatment in this direction will cause more damage to the normal healthy cells of the individual than the treatment of cancer.

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Author Contributions

Conceptualization: FÖK, Investigation: FÇS, Formal Analysis: MBB, SB, Resources: FÖK, FG, SAT Writing – Original Draft Preparation: MBB, Writing – Review & Editing: MBB, FÖK. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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References

Abbas P., Hashım Yzh., & Salleh Hm. (2019) Uninfected agarwood branch extract possess cytotoxic and inhibitory effects on MCF-7 breast cancer cells. J.Res.Pharm.; 23(1): 120-129.

http://dx.doi.org/10.12991/jrp.2018.116

Abo-Elghiet, F., Ibrahim, M. H., El Hassab, M. A., Bader, A., Abdallah, Q. M. A., & Temraz, A. (2022). LC/MS analysis of Viscum cruciatum Sieber ex Boiss. extract with anti-proliferative activity against MCF-7 cell line via G0/G1 cell cycle arrest: An in-silico and in-vitro study. Journal of ethnopharmacology, 295, 115439. https://doi.org/10.1016/j.jep.2022.115439

Alam, M. A., Quamri, M. A., Ayman, U., Sofi, G., & Renuka, B. N. (2021). Understanding Humma-e-Wabai (epidemic fever) and Amraz-e-Wabai (epidemic disease) in the light of Unani medicine. Journal of complementary & integrative medicine, 18(3), 469–476. https://doi.org/10.1515/jcim-2020-0124

Atanasov, A. G., Zotchev, S. B., Dirsch, V. M., International Natural Product Sciences Taskforce, & Supuran, C. T. (2021). Natural products in drug discovery: advances and opportunities. Nature reviews. Drug discovery, 20(3), 200–216. https://doi.org/10.1038/s41573-020-00114-z

Aziz, M.Y.A., Johari, S.A.T.T., Mamat, W.N.A.W., Taib, W.R.W., Othman, A.S., & Rohin, M.A.K. (2023) Cytotoxic Activity of Ethanolic Extract Aquilaria malaccensis Leaves Against MCF-7 Cells. *Malaysian Journal of Medicine & Health Sciences*, *19* (6), p215. https://doi.org/10.47836/mjmhs.19.6.29

Batır, M. B., Şahin, E., & Çam, F. S. (2019). Evaluation of the CRISPR/Cas9 directed mutant TP53 gene repairing effect in human prostate cancer cell line PC-3. Molecular biology reports, 46(6), 6471–6484. https://doi.org/10.1007/s11033-019-05093-y

Batir MB., Batir S., Ozdal Kurt F., & Çam S. (2023) The cytotoxic and apoptotic effects of Abies nordmanniana subsp. bornmülleriana Mattf resin extract on prostate cancer cell cells. Communications Faculty of Sciences University of Ankara Series C Biology. 2023;32(2):119-36. https://doi.org/10.53447/communc.1272043

Bertheloot, D., Latz, E., & Franklin, B. S. (2021). Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. Cellular & molecular immunology, 18(5), 1106–1121. https://doi.org/10.1038/s41423-020-00630-3

Carneiro, B. A., & El-Deiry, W. S. (2020). Targeting apoptosis in cancer therapy. Nature reviews. Clinical oncology, 17(7), 395–417. https://doi.org/10.1038/s41571-020-0341-y

Chakrabarti, G., Gerber, D. E., & Boothman, D. A. (2015). Expanding antitumor therapeutic windows by targeting cancer-specific nicotinamide adenine dinucleotide phosphate-biogenesis pathways. Clinical pharmacology : advances and applications, 7, 57–68. https://doi.org/10.2147/CPAA.S79760

Chaudhry, G. E., Md Akim, A., Sung, Y. Y., & Sifzizul, T. M. T. (2022). Cancer and apoptosis: The apoptotic activity of plant and marine natural products and their potential as targeted cancer therapeutics. Frontiers in pharmacology, 13, 842376. https://doi.org/10.3389/fphar.2022.842376

Clardy, J., & Walsh, C. (2004). Lessons from natural molecules. Nature, 432(7019), 829–837. https://doi.org/10.1038/nature03194

Dahham S. S., Ahamed M. B. K., Saghir S. M., Alsuede F. S., Iqbal M. A., & Majid A. (2014) Bioactive essential oils from Aquilaria crassna for cancer prevention and treatment. GJPAAS, 4, 26–31. https://doi.org/10.13140/2.1.3315.7125

Doak, B. C., Over, B., Giordanetto, F., & Kihlberg, J. (2014). Oral druggable space beyond the rule of 5: insights from drugs and clinical candidates. Chemistry & biology, 21(9), 1115–1142. https://doi.org/10.1016/j.chembiol.2014.08.013

Feher, M., & Schmidt, J. M. (2003). Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. Journal of chemical information and computer sciences, 43(1), 218–227. https://doi.org/10.1021/ci0200467

Guo, H., Yang, Y., Lou, Y., Zuo, Z., Cui, H., Deng, H., Zhu, Y., & Fang, J. (2023). Apoptosis and DNA damage mediated by ROS involved in male reproductive toxicity in mice induced by Nickel. Ecotoxicology and environmental safety, 268, 115679. https://doi.org/10.1016/j.ecoenv.2023.115679

Harvey, A. L., Edrada-Ebel, R., & Quinn, R. J. (2015). The re-emergence of natural products for drug discovery in the genomics era. Nature reviews. Drug discovery, 14(2), 111–129. https://doi.org/10.1038/nrd4510

Kashyap, D., Garg, V. K., & Goel, N. (2021). Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis. Advances in protein chemistry and structural biology, 125, 73–120. https://doi.org/10.1016/bs.apcsb.2021.01.003

Ketelut-Carneiro, N., & Fitzgerald, K. A. (2022). Apoptosis, Pyroptosis, and Necroptosis-Oh My! The Many Ways a Cell Can Die. Journal of molecular biology, 434(4), 167378. https://doi.org/10.1016/j.jmb.2021.167378

Kundishora, A., Sithole, S., & Mukanganyama, S. (2020). Determination of the Cytotoxic Effect of Different Leaf Extracts from Parinari curatellifolia (Chrysobalanaceae). Journal of toxicology, 2020, 8831545. https://doi.org/10.1155/2020/8831545

Lawson, A. D. G., MacCoss, M., & Heer, J. P. (2018). Importance of Rigidity in Designing Small Molecule Drugs To Tackle Protein-Protein Interactions (PPIs) through Stabilization of Desired Conformers. Journal of medicinal chemistry, 61(10), 4283–4289. https://doi.org/10.1021/acs.jmedchem.7b01120

Lee, S. O., Joo, S. H., Kwak, A. W., Lee, M. H., Seo, J. H., Cho, S. S., Yoon, G., Chae, J. I., & Shim, J. H. (2021). Podophyllotoxin Induces ROS-Mediated Apoptosis and Cell Cycle Arrest in Human Colorectal Cancer Cells via p38 MAPK Signaling. Biomolecules & therapeutics, 29(6), 658–666. https://doi.org/10.4062/biomolther.2021.143

Li, K., Zheng, Q., Chen, X., Wang, Y., Wang, D., & Wang, J. (2018). Isobavachalcone Induces ROS-Mediated Apoptosis via Targeting Thioredoxin Reductase 1 in Human Prostate Cancer PC-3 Cells. Oxidative medicine and cellular longevity, 2018, 1915828. https://doi.org/10.1155/2018/1915828

Li, X., Chen, Y., Zhao, J., Shi, J., Wang, M., Qiu, S., Hu, Y., Xu, Y., Cui, Y., Liu, C., & Liu, C. (2019). The Specific Inhibition of SOD1 Selectively Promotes Apoptosis of Cancer Cells via Regulation of the ROS Signaling Network. Oxidative medicine and cellular longevity, 2019, 9706792. https://doi.org/10.1155/2019/9706792

Li, A. X., Sun, M., & Li, X. (2017). Withaferin-A induces apoptosis in osteosarcoma U2OS cell line via generation

of ROS and disruption of mitochondrial membrane potential. European review for medical and pharmacological sciences, 21(6), 1368–1374.

Limam, I., Ghali, R., Abdelkarim, M., Ouni, A., Araoud, M., Abdelkarim, M., Hedhili, A., & Ben-Aissa Fennira, F. (2024). Tunisian Artemisia campestris L.: a potential therapeutic agent against myeloma - phytochemical and pharmacological insights. Plant methods, 20(1), 59. https://doi.org/10.1186/s13007-024-01185-4

Luna-López, A., Triana-Martínez, F., López-Diazguerrero, N. E., Ventura-Gallegos, J. L., Gutiérrez-Ruiz, M. C., Damián-Matsumura, P., Zentella, A., Gómez-Quiroz, L. E., & Königsberg, M. (2010). Bcl-2 sustains hormetic response by inducing Nrf-2 nuclear translocation in L929 mouse fibroblasts. Free radical biology & medicine, 49(7), 1192–1204. https://doi.org/10.1016/j.freeradbiomed.2010.07.004

Nahar, J., Boopathi, V., Rupa, E. J., Awais, M., Valappil, A. K., Morshed, M. N., Murugesan, M., Akter, R., Yang, D. U., Mathiyalagan, R. (2023) Protective Effects of Aquilaria agallocha and Aquilaria malaccensis Edible Plant Extracts against Lung Cancer, Inflammation, and Oxidative Stress—In Silico and In Vitro Study. Applied Sciences, 13(10):6321. https://doi.org/10.3390/app13106321

Newman, D. J., & Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. Journal of natural products, 79(3), 629–661. https://doi.org/10.1021/acs.jnatprod.5b01055

Nguyen, T. T. T., Pham, T. N. M., Nguyen, C. T. N., Truong, T. N., Bishop, C., Doan, N. Q. H., & Le, T. H. V. (2023). Phytochemistry and Cytotoxic Activity of Aquilaria crassna Pericarp on MDA-MB-468 Cell Lines. ACS omega, 8(45), 42356–42366. https://doi.org/10.1021/acsomega.3c04656

Jaramillo-Rangel, G., Chávez-Briones, M. D., Niderhauser-García, A., & Ortega-Martínez, M. (2020). Toxicity and Anticancer Potential of Karwinskia: A Review. Molecules (Basel, Switzerland), 25(23), 5590. https://doi.org/10.3390/molecules25235590

Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., & Migliaccio, A. (2020). ROS in cancer therapy: the bright side of the moon. Experimental & molecular medicine, 52(2), 192–203. https://doi.org/10.1038/s12276-020-0384-2

Prasad, S. K., Veeresh, P. M., Ramesh, P. S., Natraj, S. M., Madhunapantula, S. V., & Devegowda, D. (2020). Phytochemical fractions from Annona muricata seeds and fruit pulp inhibited the growth of breast cancer cells through cell cycle arrest at G0/G1 phase. Journal of cancer research and therapeutics, 16(6), 1235–1249. https://doi.org/10.4103/jcrt.JCRT_494_19

Raheel R., Saddiqe Z., Iram M., & Afzal S. (2017) In vitro antimitotic, antiproliferative and antioxidant activity of stem bark extracts of Ficus benghalensis L., South African Journal of Botany, Volume 111, Pages 248-257. https://doi.org/10.1016/j.sajb.2017.03.037

Rajabi, S., Maresca, M., Yumashev, A. V., Choopani, R., & Hajimehdipoor, H. (2021). The Most Competent Plant-Derived Natural Products for Targeting Apoptosis in Cancer Therapy. Biomolecules, 11(4), 534. https://doi.org/10.3390/biom11040534

Samson, F. E., & Nelson, S. R. (2000). The aging brain, metals and oxygen free radicals. Cellular and molecular biology (Noisy-le-Grand, France), 46(4), 699–707.

Sidhu, P., Shankargouda, S., Rath, A., Hesarghatta Ramamurthy, P., Fernandes, B., & Kumar Singh, A. (2020). Therapeutic benefits of liquorice in dentistry. Journal of Ayurveda and integrative medicine, 11(1), 82–88. https://doi.org/10.1016/j.jaim.2017.12.004

Solowey, E., Lichtenstein, M., Sallon, S., Paavilainen, H., Solowey, E., & Lorberboum-Galski, H. (2014). Evaluating medicinal plants for anticancer activity. TheScientificWorldJournal, 2014, 721402. https://doi.org/10.1155/2014/721402

Sytar, O., & Smetanska, I. (2022). Special Issue "Bioactive Compounds from Natural Sources (2020, 2021)". Molecules (Basel, Switzerland), 27(6), 1929. https://doi.org/10.3390/molecules27061929

Tintore, M., Vidal-Jordana, A., & Sastre-Garriga, J. (2019). Treatment of multiple sclerosis - success from bench to bedside. Nature reviews. Neurology, 15(1), 53–58. https://doi.org/10.1038/s41582-018-0082-z

Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?. Nature reviews. Drug discovery, 8(7), 579–591. https://doi.org/10.1038/nrd2803

Yau, T., Dan, X., Ng, C. C., & Ng, T. B. (2015). Lectins with potential for anti-cancer therapy. Molecules (Basel, Switzerland), 20(3), 3791–3810.