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Effects of *Crataegus azarolus* Extracts on Proliferation, Migration, and Apoptosis in AGS Gastric Cancer Cell Line

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***Abstract*—Gastric cancer remains one of the leading causes of cancer morbidity and death worldwide. Despite advances, current treatments remain costly, have low success rates, and cause adverse effects, prompting research into plant-based alternatives as a potential solution. *Crataegus azarolus* contains compounds with health potential and minimal side effects. This study was conducted to investigate the potential antiproliferative, migratory, and apoptotic effects of *C. azarolus* methanol and acetone extracts of fruits and leaves on human gastric adenocarcinoma cell lines (AGS) and human fibroblast cell lines. AGS cells were treated with different concentrations of methanol and acetone fruits (MF and AF) extracts (10, 50, 100, 250, and 500 µg/mL). Our results demonstrated that MF extract showed significantly higher anti-proliferative activity at 500 µg/mL concentrations, when estimated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay ($p < 0.001$). The calculated half-maximal inhibitory concentration value was 300 µg/mL. Furthermore, MF extract showed a significant ($p < 0.01$) cellular migration inhibitory effect. The clonogenicity of AGS was considerably inhibited ($p < 0.001$), resulting in a decrease in holoclone production in AF and acetone leave (AL) extracts. Flow cytometry revealed the induction of apoptosis, with the highest 31.4% MF. Early growth response-1 expression increased dramatically ($p < 0.001$) in AF extract, while enhancer of zeste homolog-2 expression increased significantly ($p < 0.001$) when treated with MF and AL. Phosphatase and tensin homolog and N-myc downstream regulated gene-1 were both highly upregulated in MF and AF extracts, respectively. In conclusion, *C. azarolus* demonstrated anti-cancer effects on the AGS cell line and may have potential as a source of anticancer compounds.**

***Index Terms*—Anti-proliferative, Apoptosis, Crataegus extracts, Early growth response-1, Gastric cancer, Human adenocarcinoma gastric.**

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I. Introduction

Cancer is the second leading cause of death worldwide, with incidence rates continuously increasing over recent decades (Hussain and Lafta, 2021) and projected to exceed 17 million deaths by 2030 (Omairi, Kobeissy, and Nasreddine, 2020). In Iraq, despite anti-cancer initiatives since 1974, incidence and mortality remain high (M-Amen, et al., 2022) due to limited prevention efforts and increasing risk factors such as diabetes, obesity, poor diet, and smoking (Hussain and Lafta, 2021; Barzani, et al., 2025)

Gastric cancer (GC) is among the most frequent cancers globally (Wong, et al., 2021; Guan, He and Xu., 2023). Limited data exist on recent cancer incidence in Iraq's Kurdistan Region, but statistics show that cancer cases doubled between 2013 and 2019 (M-Amen, et al., 2022). Notably, natural plant compounds have been a subject of interest as they are likely to be less harmful than synthetic ones in terms of side effects. One example is *Crataegus azarolus*, commonly known as hawthorn (Zurek, et al., 2021). Conventionally, various parts of this shrub, including fruits, leaves, flowers, and bark, have been used to treat stomach and colon pains and other conditions due to their antioxidant, antibacterial, antifungal, antidepressant, and anti-inflammatory properties (Rastogi, Pandey and Rawat, 2016; Niu, et al., 2020). Compounds derived from *Crataegus* genus have been verified for their capabilities to reduce the growth of various cancer cell lines through multiple mechanisms. The main targeted mechanism is apoptosis (Shimizu, et al., 2014). As a result, activating apoptotic pathways has become a key focus for investigating and enhancing the medicinal effectiveness of new drugs, particularly in cancer treatment (Indran, et al., 2011).

Early growth response-1 (EGR1) has a dual function in different cancer types and is a direct regulator for different tumor suppressor genes (Mohamad, et al., 2018). Inhibition of phosphatase and tensin homolog (PTEN) protein activity has been identified in many GC cases, and loss of function is strongly related to disease prognosis (Zheng, et al., 2018b). The *PTEN* gene has positive regulators that directly link

to the PTEN promoter region, such as EGR1 and p53 (Xu, yang and Lu, 2014). Enhancer of zeste homolog-2 (EZH2) expression has been linked to the suppression of tumor suppressor gene activity (Chen, et al., 2015). Research has shown that different plant extracts can impair disease development and reduce tumor formation in various cancers; however, the underlying mechanisms of these effects regarding specific genes are still not fully understood (Sun, et al., 2018).

Although many studies have examined the anti-cancer effects of hawthorn components, little is known about their impact on the stomach. This study aimed to assess the effects of *C. azarolus* fruit and leaf extracts on the GC cell line.

II. Materials and Methods

A. Plant Collection and Extracts Preparation

C. azarolus plant, fresh fruits, and leaves were collected in August 2021 from Safin Mountain, Erbil-Shaqlawa, in the Kurdistan Region, Iraq. It was then identified and authenticated by the Biology Department, College of Science, Salahaddin University, Erbil. Methanol and acetone extracts of dried fruits and leaves were prepared as described previously by Mohammedsaeed and Mohamad, 2023. The samples were preserved in −20°C.

Phytochemical screening

After the extraction process, qualitative phytochemical screenings were performed to identify and classify the compounds present in the extracts of *C. azarolus* fruits and leaves. These screenings employ a set of chemical methods that detect the chemical compounds produced by plants, as described by Harborne, 1984; Mohammedsaeed and Mohamad, 2023.

Conditions of cell lines and cell culture

The AGS and normal fibroblast cell line were obtained from the Pasteur Institute, Iran. Both cell lines were cultured and maintained as a monolayer in F-12 medium with 10% fetal bovine serum, and antibiotics (100 U/mL; Gibco, USA) streptomycin, and (100 U/mL; Gibco, USA) penicillin. Cells were cultured in a humidified incubator with 5% carbon dioxide at 37°C. The culture medium was replaced every 2–3 days.

Cell viability assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT])

The anti-proliferative effects of varying concentrations of *C. azarolus* fruit and leaf extracts on AGS cells were assessed using the MTT assay, which measures cell viability through formazan production by metabolically active cells (Mustapha, et al., 2015). 96-well plates were seeded with 10,000 AGS cells and normal fibroblast cell lines per well, and the plates were cultured for 24 h. Subsequently, the cells were exposed to various concentrations of *C. azarolus* extracts (10, 50, 100, 250, and 500 μg/mL). After 48 h, the medium was removed, and 100 μL of MTT reagent (Sigma Aldrich, USA) was added to each well. The cells were then incubated for an additional 2–4 h. The medium was discarded, and 100 μL of dimethyl sulfoxide was added to the wells. Cell viability was measured at 540 nm using a microplate enzyme-linked immunosorbent assay reader (DA3200, Iran).

Wound healing assay (scratching assay)

Scratching assay was performed to determine the cells' capacity for migration after introducing a wound. The results were obtained by measuring the wound distance at 0 h, 24 h, and 48 h, and comparing them to the distance at zero time for each time independently (Omairi, Kobeissy and Nasreddine, 2020).

The scratch assay was conducted on normal fibroblast and AGS cells treated with half-maximal inhibitory concentration (IC_{50}) concentrations of *C. azarolus* extracts (methanolic: 300 μg/mL fruits, 250 μg/mL leaves; acetone: 350 μg/mL fruits and leaves). After cells reached 90% confluence, scratches were created using a 10 μL pipette tip, washed with phosphate-buffered saline (PBS), and observed at 0, 24, and 48 h using a CKX41 inverted microscope. Wound closure was quantified with ImageJ software. The results at each time point were measured and compared to time zero using the formula (scratch distance at time zero – wound distance at different time points). These results were then compared to the untreated control cells.

Colony formation assay

The colony formation assay, also referred to as the clonogenic assay, is used in vitro to assess a single cell's capacity to proliferate into a colony (Franken, et al., 2006).

A total of 2×10^5 cells were treated with different concentrations of *C. azarolus* extracts, 300 μg/mL of methanol fruit (MF), 250 μg/mL of methanol leave (ML), 350 μg/mL of acetone fruit (AF), and 350 μg/mL of acetone leave (AL). An untreated control group was also included. In both the treatment and control groups, cells were then separated into individual cells using trypsinization after 2 days. Next, 2×10^2 cells were plated in 35 mm dishes for 2 weeks with media changes every 2 days. Then, the media were discarded, and cells were gently rinsed with PBS. They were later fixed and stained by adding a mixture of 4% paraformaldehyde and 0.3% crystal violet (3 mL/plate) and allowing it to stand for 5 min. Subsequently, the stain was gently rinsed off with tap water.

Colonies were assessed quantitatively and qualitatively (holoclone, meroclone, paraclone) using an Olympus inverted microscope (×10) and counted with ImageJ software.

RNA-extraction and reverse transcription

RNA was extracted using the total RNA isolation kit (Azist Asia Total RNA isolation kit, Iran). RNase-free DNase was added to eliminate contaminating DNA. The Easy™ complementary deoxyribonucleic acid (cDNA) Synthesis Kit (Parstuos, Iran) was used to reverse transcribe the extracted RNA into cDNA following the manufacturer's instructions.

Quantification of gene expression

Relative expression of target genes was assessed using the Applied Biosystems Step One Real-Time Polymerase Chain Reaction (PCR) system (Lincoln Central Drive, Foster City, USA), with SYBR Green Master Mix (Amplicon RealQPlus ×2, High ROX™, Denmark). Gene of interest expressions were analyzed in comparison to the beta-actin gene as an

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internal reaction control. Negative controls were involved, where no reverse transcriptase was added. Expression levels were normalized to beta-actin as the reference gene. Reverse transcription quantitative PCR data were calculated using the comparative threshold cycle (Ct) method. Standard deviations (SD) from the mean of the ΔCt values were calculated from at least two independent RNA samples. Fluorescent probes based on SYBR Green were used with the primers as listed in Table I. The PCR cycling conditions were as follows: 5 min at 95°C, then 40 cycles using a step program that included 30 s at 95°C, 20 s at 60°C, and 20 s at 72°C.

Flow cytometry for cell cycle analysis (apoptosis)

Cells were processed and incubated for 48 h to analyze the cell apoptosis rate under the effect of one-time IC_{50} concentrations of *C. azarolus* extracts. Adherent cells were subjected to trypsinization for 2 min, followed by neutralization, and then harvested and centrifuged at 12000 rpm, 25°C for 5 min. Cells were resuspended in 500 µL of 1× Annexin V Binding Buffer. Next, they were treated with a mixture of 10 µL Annexin V-FITC and 5 µL propidium iodide and kept at room temperature in the dark for 15 min. Finally, the cells were analyzed using a FACSCalibur Flow cytometer.

Statistical analysis

Each experiment was conducted 3 times independently in triplicate. One-way analysis of variance, the Brown-Forsythe test, and GraphPad Prism 8.0.2 (San Diego, USA) were used for the statistical analysis. The mean ± SD is used to represent the results. The arithmetic means ± SD of three separate experiments are described in the data. Probability values (p) < 0.05, 0.01, and 0.001 were used to define statistical significance, and significance thresholds were established in accordance with these criteria.

III. RESULTS

A. Phytochemical Screening of C. azarolus Fruit Extracts Revealed the Presence of Numerous Bioactive Compounds

Phytochemical screening tests of *C. azarolus* fruits' crude extracts indicated the existence of some significant bioactive components, as shown in Table II. This phytochemical study was qualitatively expressed as positive (+) or negative (−) to denote the presence. All results are mentioned in Table II.

TABLE I
PRIMER SEQUENCES USED FOR QRT-PCR

Genes		Primers	Reference
EGR1	Forward	5'TTCCTCAGCTGTCACCAACTC3'	Cinacolon company
	Reverse	5'CTCTGAGAACCTCCATCTGACC3'	
EZH2	Forward	5'GACCTCTGTCTTACTTGTGGAGC3'	
	Reverse	5'CGTCAGATGGTGCCAGCAATAG3'	
NDRG1	Forward	5'TGGTTCCAGCGTCACTTCTC3'	
	Reverse	5'ACCGAGTTAGGCGCAGTATG3'	
PTEN	Forward	5'ACCAGGACCAGAGGAAACCT3'	
	Reverse	5' GCTAGCCTCTGGATTTGACG3'	
p21	Forward	5'CCAGCATGACAGATTTCTACCAC3'	
	Reverse	5'GGCCAGGGTATGTACATGAGGAG3'	
Beta-ACTIN	Forward	5'CAGGTGGTCTCCTCTGACTTCAAC3'	
	Reverse	5'AGGGTCTCTCTCTTCCTCTTGTGC3'	

QRT-PCR: Quantitative reverse transcription polymerase chain reaction, EGR1: Early growth response-1, EZH2: Enhancer of zeste homolog-2, NDRG1: N-myc downstream regulated gene-1, PTEN: Phosphatase and tensin homolog

B. Anti-proliferative Properties of C. azarolus MF Extract

The AGS and normal fibroblast cell lines were treated with serial concentrations of MF (10, 50, 100, 250, and 500 µg/mL), respectively. Our results (Fig. 1a) show that MF had significant cytotoxic (inhibitory) influences on AGS proliferation in a dose-dependent manner after 48 h, where IC_{50} value was 300 µg/mL. After exposure to an increasing concentration of MF at 500 µg/mL, there was a significant ($p < 0.001$) decrease in AGS cell proliferation. In contrast, lower concentrations (10, 50, 100, and 250 µg/mL) had no significant inhibitory effects on AGS cells' viability.

On the other hand, as shown in Fig. 1b, MF showed significant cytotoxic effects on normal fibroblast cells proliferation, where the IC_{50} value was 300 µg/mL. After exposure to an increasing concentration of MF at 50 µg/mL, there was a significant ($p < 0.05$) increase in normal fibroblast cell proliferation. Then with increasing doses (250 and 500 µg/mL) respectively, the number of viable normal fibroblast cells significantly declined ($p < 0.01$ and $p < 0.001$) relative to untreated control cells. Concentrations (10 and 100 µg/mL) had no significant effects on cell viability.

C. AF Extract Effects on AGS and Normal Fibroblast Cell Lines

The AGS and normal fibroblast cell lines were treated with different concentrations of AF (10, 50, 100, 250, and 500 µg/mL) for 48 h. Results in (Fig. 2a) show that with the AF, only 500 µg/mL revealed a significant ($p < 0.001$) effect in reducing AGS cells viability relative to the untreated control. IC_{50} value for AF on AGS cell proliferation after 48 h of incubation was 350 µg/mL. On the other hand, AF showed significant cytotoxic effects on normal fibroblast cells proliferation as depicted in (Fig. 2b). After exposure to an increasing concentration of AF, only 500 µg/mL recorded a significant ($p < 0.001$) effect, reducing the viability of normal fibroblast cells relative to untreated control. Lower concentrations (10, 50, 100, and 250 µg/mL) showed no significant effects on normal fibroblast cells' viability.

TABLE II
PHYTOCHEMICAL SCREENING OF *CRATAEGUS AZAROLUS* FRUITS EXTRACTS

No.	Chemical components	Fruits	
		Methanol	Acetone
1	Alkaloids	+++	+++
2	Polyphenol (Tannins)	++	+++
3	Polyphenol (Flavonoid)	+	+++
4	Triterpenoid	−	−
5	Peptides and free amino group	−	−
6	Carbohydrates	+++	+++
7	Glycosides	+++	++
8	Protein	−	−
9	Saponins	++	++

+: Low in concentration, ++: Moderate, +++: High, −: Absent

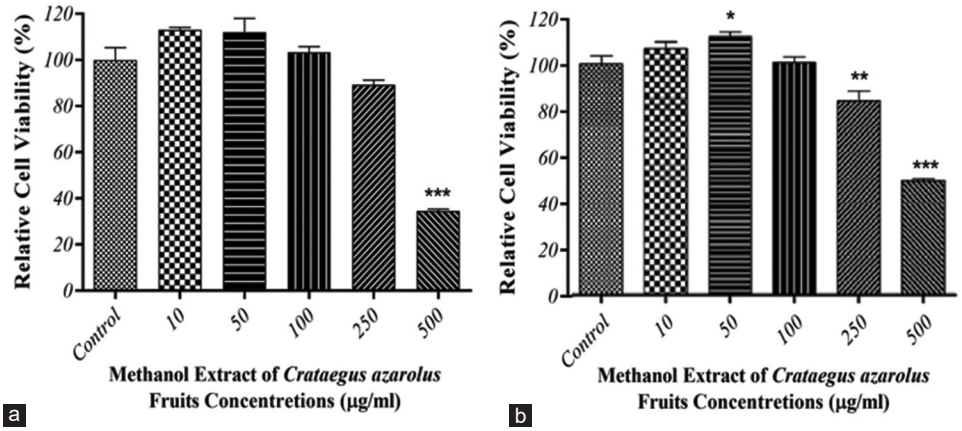

Fig. 1. Anti-proliferative effects of *Crataegus azarolus* methanol fruit (MF) extract. (a) The viability of AGS cell line. (b) The viability of normal fibroblast cell line. After 48 h of treatment with different concentrations of MF extract relative to untreated control, estimated by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (*p < 0.05, **p < 0.01, and ***p < 0.001).

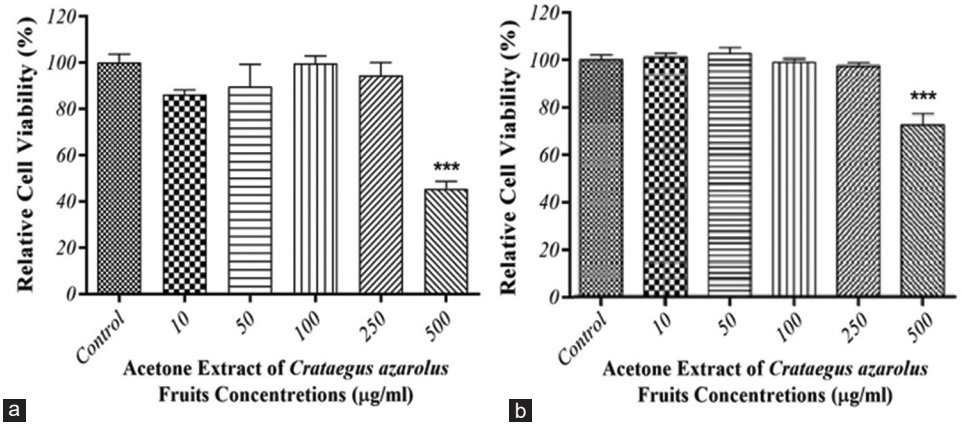

Fig. 2. Anti-proliferative effects of *Crataegus azarolus* acetone fruits (AF) extract. (a) The viability of AGS cells. (b) The viability of normal fibroblast cell line. After 48 h of treatment with different concentrations of AF relative to untreated control, measured by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (***p < 0.001).

D. C. azarolus Extracts Inhibited Cell Migration, Performing a Wound Healing Assay

The effects of the *C. azarolus* extracts on AGS and normal fibroblast cells migration were analyzed using the scratching assay. The images of scratch area wounds were taken at different time intervals, which were 0 h, 24 h, and 48 h, as shown in (Figs. 3 and 4). The effects of the extracts on cells migration after 24 h showed that all tested extracts had no inhibitory effects on the migration of AGS cells compared to the control. Moreover, after 48 h of treatment, MF showed significant ($p < 0.01$) inhibitory effects on AGS cell migration compared to the control. AL exhibited an inhibitory effect on the migration of AGS cells, but it was not substantial. Whereas, both ML and AF had no impacts on AGS cells migration, as shown in Fig. 3.

On the other hand, the effects of the investigated extracts on the migration of normal fibroblast cells after 24 h treatment were as follows; our results showed that MF significantly decreased ($p < 0.001$) the migration of normal fibroblast cells, whereas AL did not inhibit the migration of normal fibroblast cells, although it was not significant. After 48 h, MF and AL had a significant ($p < 0.001$) inhibiting effect on normal fibroblast cell migration, while AF had a non-significant effect compared to the control (Fig. 4).

E. Colony Formation Assay Explained the Anti-colonogenic Effects of the Extracts and Holoclone Reduction

Clonogenic ability of AGS cells treated with extracts for 2 weeks compared to untreated control cells (Fig. 5). The results showed a prominent clonogenic property. The number and type of clones decreased significantly after 2 weeks of treatment with extracts. There were significant differences between the treatments in terms of the number of clones formed and their types. MF showed the highest holoclone type 73% compared to the other treatments, whereas the lowest 15% was observed in AL. The highest paraclone rate was recorded in ML 39%, while the lowest rates were recorded in MF at 7%. Moreover, the highest meroclone in MF 20% was compared to the lowest, which was detected in both AF and AL, 29% and 43%, respectively. While the untreated control showed the highest holoclone 72%, compared to the meroclone 23% and paraclone 4%. Full results are presented in Table III.

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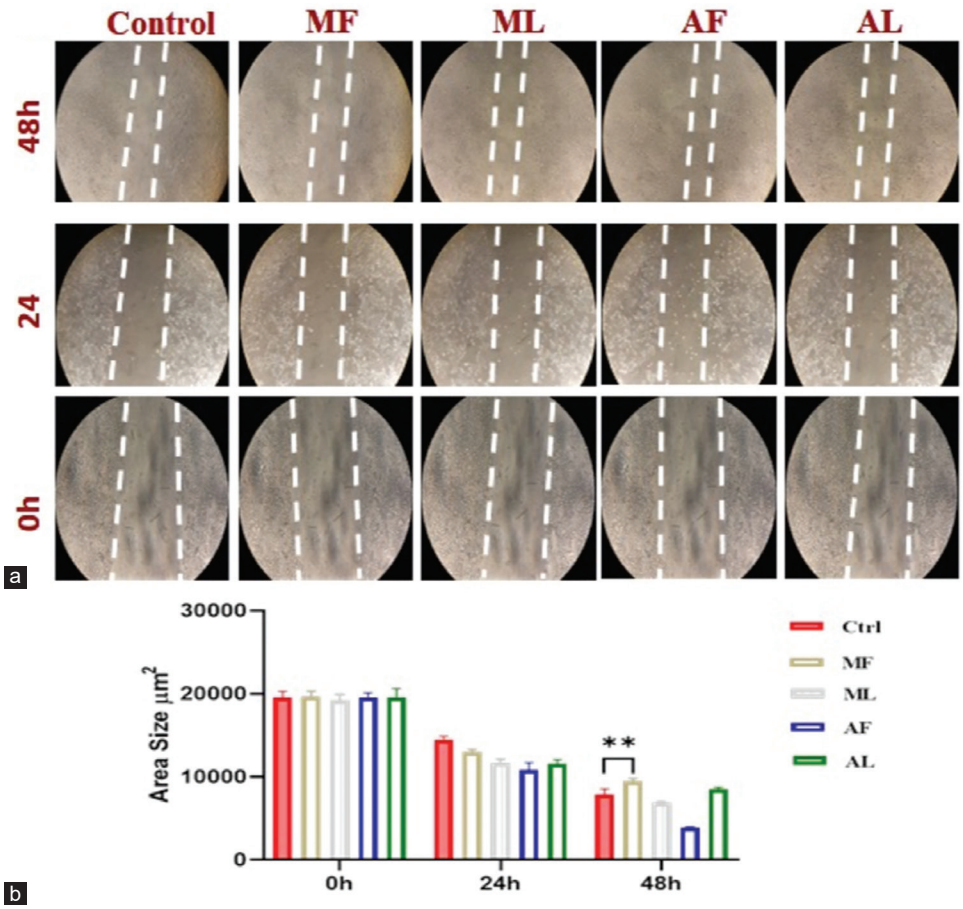

Fig. 3. Migration inhibition effect of *Crataegus azarolus* extracts on AGS cells by wound healing assay. (a) Photomicrographs were taken immediately after wound healing at the time point 0, 24, and 48 h. Images were taken at ×10 magnification. Representative photomicrographs of one out of three independent experiments are shown. (b) Effect of extracts on invasive potential of AGS cells. Distance plotted against time point of each sample (***$p < 0.001$). AF: Acetone fruits, AL: Acetone leaves, MF: Methanol fruits, ML: Methanol leaves.

F. Quantitative Gene Expression Modulations were Detected

The reference gene, beta-actin, was used to determine the relative expression of the target genes. The expression levels of all the genes under study were compared to the untreated control (Fig. 6). The outcomes from the AGS cells treated with MF of *C. azarolus* indicated that the expression of p21, EGR1, and N-myc downstream regulated gene-1 (NDRG1) was upregulated, although the changes were not statistically significant. Whereas both PTEN and EZH2 revealed significant upregulation ($p < 0.01$ and $p < 0.001$, respectively). ML extract results showed that p21, EGR1, EZH2, and NDRG1 were upregulated, but with no significant effects. While PTEN expression was downregulated. AF results showed a significant upregulation ($p < 0.001$) of EGR1 and NDRG1, whereas p21, PTEN, and EZH2 were also upregulated, but not significantly. The results from AL demonstrated that only EZH2 showed significant upregulation ($p < 0.001$) in expression, while the other genes, p21, PTEN, EGR1, and NDRG1, showed no significant upregulation.

G. Extracts Trigger AGS Cells toward Apoptosis

The AGS cells treated with *C. azarolus* extracts were analyzed using flow cytometry to measure the degree of apoptosis activity after 48 h of treatment. The scatter graph was set up with four quadrants, namely Q1 (dead cells), which were necrotic cells, Q2 (late apoptosis), Q3 (early stages of apoptosis), and Q4 (live cells). To calculate the overall apoptotic cell percentage found during the assay, the data of dead apoptotic cells recorded in the Q2 and Q3 were combined. Levels of apoptosis were 16.8% (untreated AGS cells, control), 31.4% MF, 16% ML, 37.9% AF, and 20.6% AL.

The analysis of untreated AGS cells revealed that 63.3% of cells were located in Q4 and remained viable, while 36.6% of cells died (necrosis, early apoptosis, and late apoptosis) with 19.8% in Q1, 13.6% in Q2, and 3.2% in Q3 (Fig. 7a). Cells that were treated with MF indicated that 28.8% of cells located in Q4 remained viable; 71.1% cell death was also observed with 39.7% cells in Q1, 26.5% in Q2, and 4.9% in Q3. Whereas cells treated with ML indicated that 32.7% remained viable in Q4, 67.3% of the cells died with 51.3% in Q1, 11.6% in Q2, and 4.3% in Q3. AGS cells treated with AF showed 42.8% viable cells in Q4; 57.1% cell death was observed with 19.2% in Q1, 25.4% in Q2, and 12.5% in Q3. The AGS cells treated with AL recorded 33.6% viable cells in Q4, 66.4% cells death observed, comprising 45.7% in Q1, 17.9% in Q2, and 2.6% in Q3 (Fig. 7b).

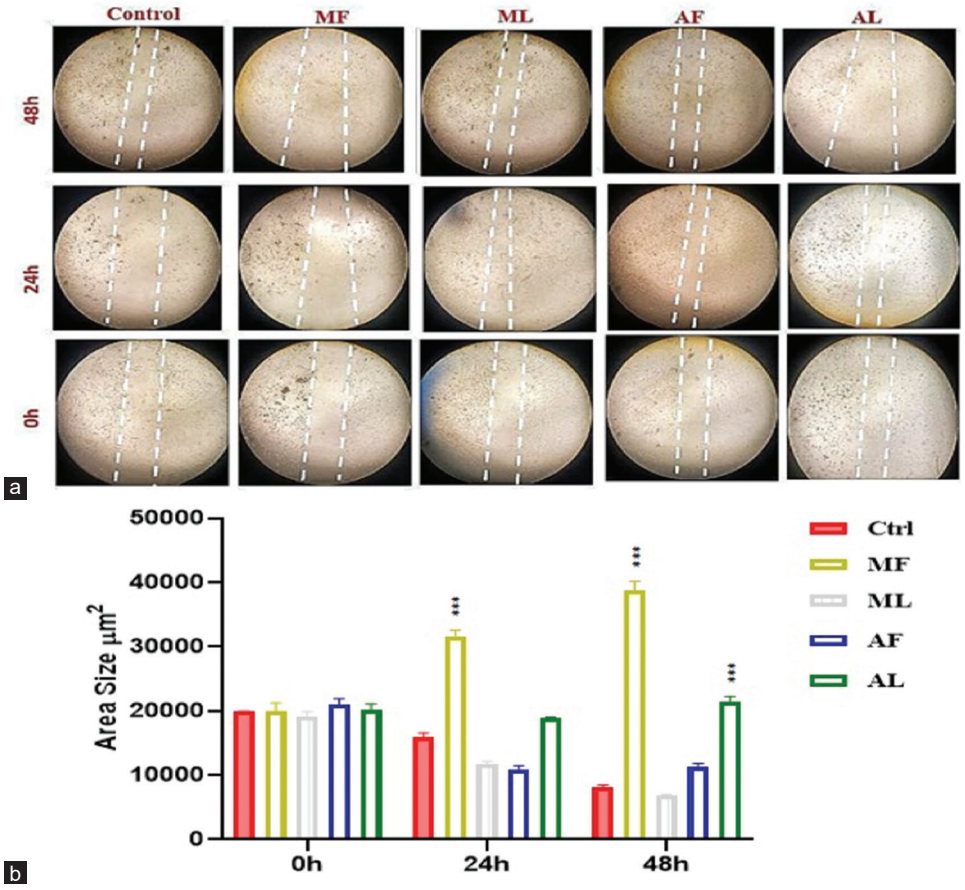

Fig. 4. Migration inhibition effect of *Crataegus azarolus* extracts on normal fibroblast cells by wound healing assay. (a) Photomicrographs were taken immediately after wound healing at the time points 0, 24, and 48 h. Images were taken at ×10 magnification. Representative photomicrographs of one out of three independent experiments are shown. (b) Effect of extracts on invasive potential of normal fibroblast cells. Distance plotted against time point of each sample (***$p < 0.001$). AF: Acetone fruits, AL: Acetone leaves, MF: Methanol fruits, ML: Methanol leaves.

IV. Discussion

Our previous work demonstrated that methanol and acetone leaf extracts exhibited stronger dose-dependent anti-proliferative effects on AGS cells compared to the control, likely due to solvent differences (Mohammedsaeed and Mohamad, 2023). These data provide the first evidence that *C. azarolus* possesses cytotoxic effects.

Phytochemical assays revealed that both methanolic and acetone extracts contain key components that may exert cytotoxic effects on various cancer cells. The highest amounts of polyphenols, which include tannins and flavonoids, were found in AF extract, whereas a lesser amount was found in MF extract. These results align with the outcomes reported by (Lakache, et al., 2016; Deveci, et al., 2020). *Crataegus* spp. berries, leaves, and flowers are rich in phenolics and flavonoids, with varying concentrations depending on solvent polarity used in extract preparation, and these compounds contain hydroxyl groups that scavenge-free radicals, protecting cells from oxidative damage and reducing cancer risk (Lakache, et al., 2016, Sulaiman and Demir, 2023). A glycoprotein isolated from *Laminaria japonica* demonstrated anti-cancer activity in AGS cell line (Huang, et al., 2020). Alkaloids have been found to exhibit cytotoxic effects on various cancer cells, including colon cancer (T84 and Cac0-2), prostate cancer (DU145), and ovarian (CAOV-3), as agents that induce apoptosis in cancerous cells (Habli, et al., 2017). Our results revealed a high amount of alkaloids in MF, AF, and ML, whereas they were found in a lesser amount in AL. These alkaloids may also have cytotoxic effects on AGS cells. MF significantly reduced AGS cell proliferation in a dose-dependent manner, likely due to their high phytochemical content. In contrast, MF revealed lower toxicity on normal fibroblasts, with only slight proliferation reduction at higher concentrations (500 µg/mL). Furthermore, the IC_{50} values were higher in normal cells than in AGS cells. Higher concentrations of the phytochemicals can also affect the viability of normal cell, however, normal cells generate lesser amounts of reactive oxygen species compared to tumor cells, and thus this could protect the normal cells from being targeted by phytochemicals (Majrashi, et al., 2023).

The AF showed a significantly higher decrease in cell proliferation compared to the leaves extract. Considering the effects of fruit and leaf acetone extracts on normal fibroblast cells showed no pronounced effect on cell viability. Similar effects were observed in other cancer cell lines, where the implied mechanism may be due to apoptosis induction. This might explain why, in the present study, the proliferations of AGS cells decreased with increasing *C. azarolus* concentrations.

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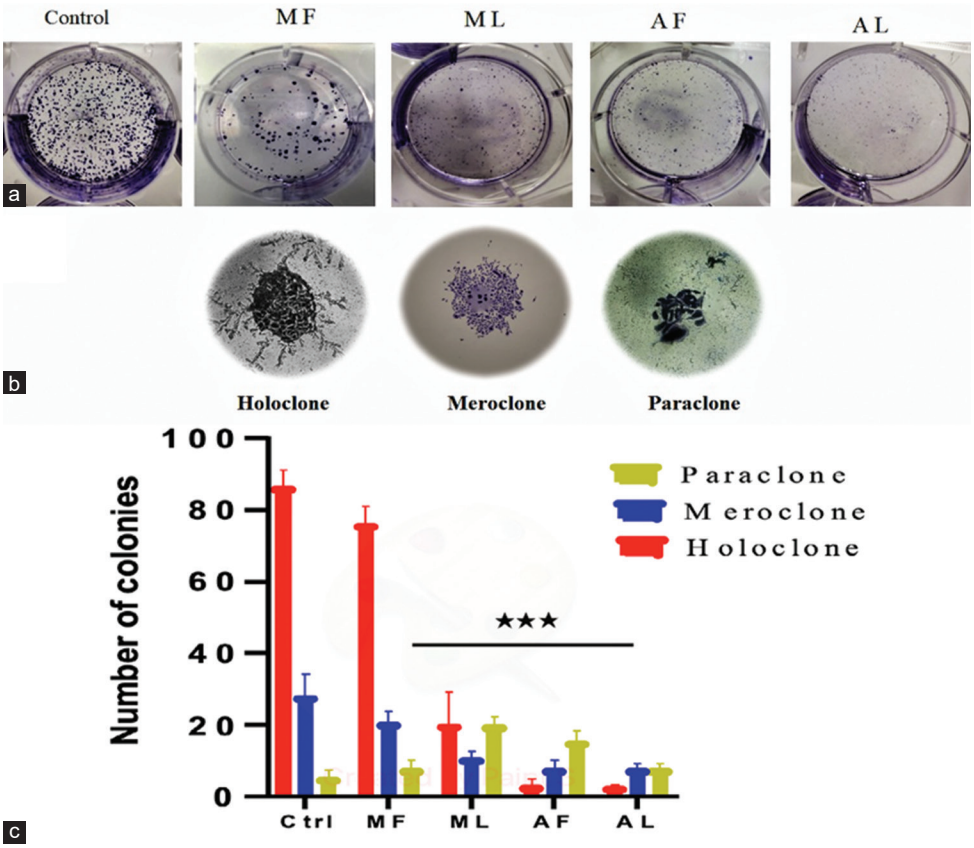

Fig. 5. Colony formation assay of AGS cells treated with *Crataegus azarolus* extracts. (a) Colony formation of the AGS cell after treatment with MF, MF, AF, and AL extracts, relative to the untreated control. (b) Types of clones formed by treated AGS cells. (c) Number of clones of treated AGS and untreated control cells (***p < 0.001). AF: Acetone fruits, AL: Acetone leaves, MF: Methanol fruits, ML: Methanol leaves.

TABLE III
TYPES, NUMBER, AND PERCENTAGES OF CLONES FORMED IN AGS CELLS TREATED WITH *CRATAEGUS AZAROLUS* EXTRACTS

Type of clones extracts	Holoclone		Meroclone		Paraclone		Total number
	Number	Percentage	Number	Percentage	Number	Percentage	
Control	86	72	28	23	5	4	120
MF	76	73	21	20	8	7	104
ML	20	40	11	21	20	39	50
AF	3	12	8	29	15	59	26
AL	2	15	8	43	8	43	18

Ags: Human gastric adenocarcinoma cell lines, MF: Methanol fruit, ML: Methanol leave, AF: Acetone fruits, AL: Acetone leave

Metastasis occurs when the adherence of cancer cells to each other is disrupted, allowing them to invade other body organs. Despite research progress, effective therapies remain limited due to resistance and adverse effects (Omairi, Kobeissy and Nasreddine, 2020). The results of the scratching assay elucidated that all extracts, after 24 h of treatment, had no significant impact on AGS cells' migration. Whereas, after 48 h of treatment, MF showed a significant migration inhibitory effect. AL showed a mild effect, which was not significant compared to the control. The migration inhibition could be boosted if fractions of the most potent known *C. azarolus* components are used. While in normal fibroblast cells, after 48 h of treatment, only MF and AL inhibited migration, AF also showed an inhibitory effect compared to the control. The above results suggest that *C. azarolus* extracts may contribute to anticancer research as a potential source for GC treatment due to their anti-proliferative and anti-migration effects.

Cancer cells tend to grow in colonies that come into contact with neighboring cells; loss of contact with neighboring cells leads to the death of cancer cells. The findings of the current study revealed that all extracts efficiently blocked one of the pivotal characteristics required by cancer cells for continuous growth and proliferation and forming colonies, thereby facilitating the establishment of two critical cancer hallmarks, migration and metastasis. In this study, all extracts could significantly block colony formation of AGS cells.

Investigating the colony formation ability was expanded to seek the types of clones in addition to their numbers. Holoclones have the greatest reproductive capacity under standard conditions; only <5% of colonies formed by holoclone cells abort and terminally differentiate, making

it one of the most challenging types of colonies. Paraclone contains cells characterized by a short reproductive lifespan of no more than 15 cell generations, after uniformly aborting and terminally differentiating. Whereas the meroclone is a transitional stage between the holoclone and paraclone, it includes a range of cells with different growth potentials (Barrandon and Green, 1987; Castellón, et al., 2012). Here, we revealed that ML, AF, and AL significantly ($p < 0.001$) decreased holoclone and meroclone compared to the untreated control. Whereas MF elucidated no significant decreases in both holoclone and meroclone. While paraclone showed no significant increase in all extracts compared to the control.

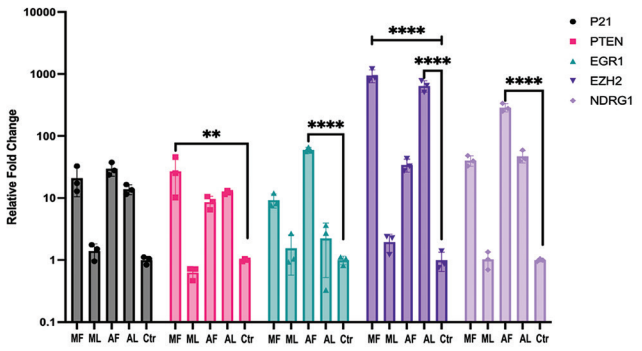

Fig. 6. Transcriptional profiles were measured by reverse transcription polymerase chain reaction for genes p21, p21, PTEN, EGR1, EZH2, and NDRG1 in AGS cells treated with half-maximal inhibitory concentration of MF, ML, AF, and AL extracts. All expression values of all studied genes were compared to untreated control (**$p < 0.01$, and ***$p < 0.001$). AF: Acetone fruits, AL: Acetone leaves, MF: Methanol fruits, ML: Methanol leaves, PTEN: Phosphatase and tensin homolog, extracts, EGR1: Early growth response-1, EZH2: Enhancer of zeste homolog-2, NDRG1: N-myc downstream regulated gene-1.

In this study, AF and AL showed the best inhibitory effect on holoclones compared to the untreated control, which may prove the cytotoxic effects of the bioactive components, including tannins and flavonoids, within these two extracts. Comparable effects on the MCF7 cell line were obviously observed, and the percentage of holoclones declined in another study (Wang, et al., 2014).

To assess the mechanism behind cell proliferation reduction, apoptosis was studied, and extracts were tested on AGS cells. Extracts can induce cell cycle arrest and regulate key signaling pathways involved in cell death, such as p38 MAPK and PI3K/AKT/mTOR pathways, via both intrinsic and extrinsic mechanisms (Ma, et al., 2020). Flow cytometry suggests that bioactive molecules in the extracts may induce apoptosis, disrupting cell cycle regulation. Thus, the anti-proliferative effects of *C. azarolus* extracts on AGS cells likely occur through apoptosis, independent of the EGR1 pathway.

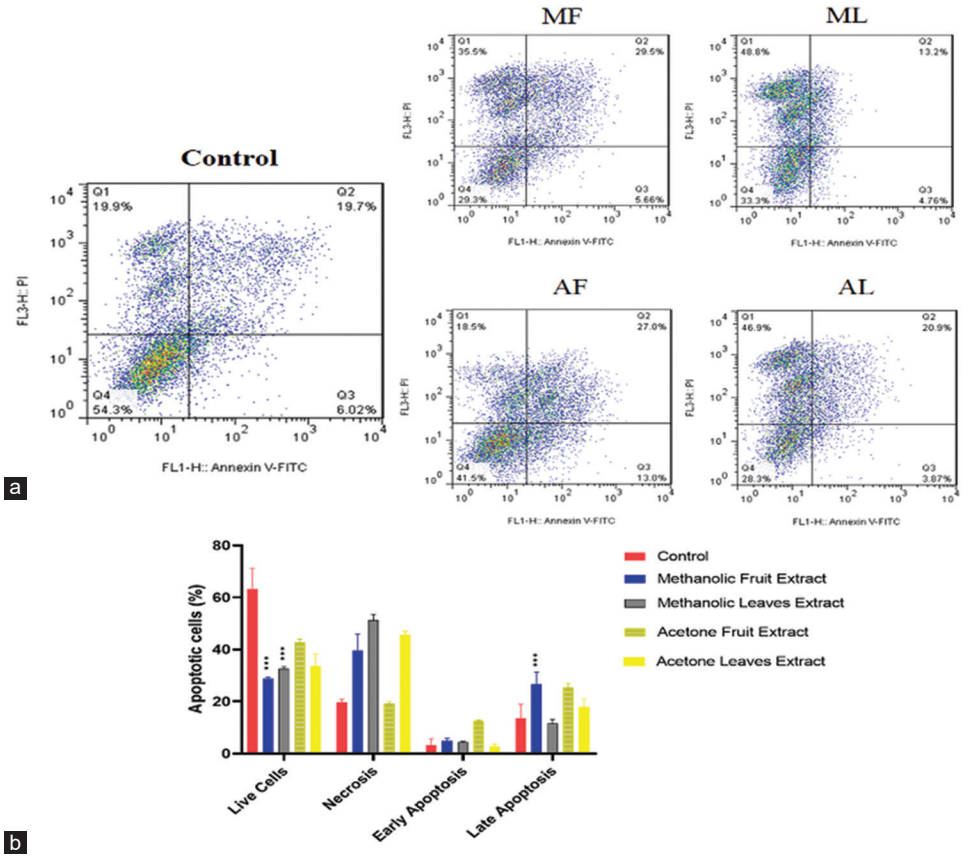

Fig. 7. Apoptotic effects of *Crataegus azarolus* extracts on AGS cells measured by using flow cytometry. (a) Scatter graph of AGS cells, Q1 (necrosis), Q2 (late apoptosis), Q3 (early apoptosis), and Q4 (live cells). Representative Scatter graphs of one out of three independent experiments are shown. (b) statistical representation of apoptotic cells after treatment in comparison to untreated cells. Plots in (a) show results of apoptotic studies of untreated cells of AGS together with cells treated with methanol fruit, methanol leave, acetone fruits, and acetone leave extracts, respectively.

Where abnormal expressions of different genes might be attributed to carcinogenesis approaching different mechanisms and targeting several signaling pathways, the current study investigated the influence of the extracts on a number of different genes. According to many studies, it has been demonstrated that upregulation of EGR1 and EZH2 plays a role in GC progression.

Linc01503, a lncRNA linked to cancer progression, is highly elevated in GC and transcriptionally activated by EGR1. By interacting with EZH2 and LSD1, it epigenetically suppresses the expression of dual-specificity cyclin-dependent kinase. Knockdown of Linc01503 induces apoptosis in GC cells (Ma, et al., 2021). EGR1 expression was increased in GC, and inhibited the apoptosis in different GC cell lines, SGC-7901, BGC-823, and MKN-45, while silencing EGR1 expression promoted apoptosis (Yang, et al., 2019). EZH2 levels were markedly up-regulated in GC tissues compared to adjacent normal tissues. It was also demonstrated that knockdown of EZH2 via sh-EZH2 in GC cell lines showed anti-proliferation effect and promoted apoptosis (Xu, Lai and Hua, 2019b). In the current study, it was elucidated that extracts had mediated anti-proliferative effects on AGS cells through different pathways, which means that the extracts' bioactive components might block the role of both EGR1 and EZH2 as oncogenes in GC, despite their expression being upregulated. In this regard, this study might contribute to the discovery of new potential molecules in *C. azarolus* extracts.

It has been observed that PTEN and NDRG1 were significantly up-regulated. Whereas an elevation of p21 expression was also observed, albeit not significantly, this could indicate pronounced effects on gene expression in GC cells. The non-significant upregulation of p21 may be due to the upregulation of EGR1 and EZH2 in the treated AGS cells. According to other studies, EGR1 inhibits the expression of p21 through induction of CDC34-mediated ubiquitination and degradation of p21 (Liu, et al., 2018). On the other hand, EZH2 binds to the p21 promoter and induces H3K27me3 modification, which results in silencing of the p21 transcription and tumorigenesis development in GC (Xu, et al., 2019a). Chromatin Immunoprecipitation assay is required for detecting EZH2 binding to p21 promoter in AGS cells.

It has been observed that EGR1 directly controls the expression of several tumor suppressor genes, including p53, PTEN, and NDRG1 (Mohamad, 2017; Mohamad, et al., 2018). NDRG1 functions in inhibiting various oncogenic signaling pathways, which are associated with declining apoptosis, proliferation, invasion, angiogenesis, and metastasis. NDRG1 is closely associated with adherent junctions and supports the development of the E-cadherin/catenin complex. In GC, increased H3K27me3 activity downregulates NDRG1, disrupting tight junctions and encouraging tumor aggressiveness (Xiao and Zheng, 2020). MF and AF significantly up-regulated the expression of PTEN and NDRG1, which could interfere with the oncogenes' role, EGR1 and EZH2, in inhibiting the apoptosis mechanism in AGS. AF significantly upregulated NDRG1 expression, likely due to the mechanical effects of sonication. By inducing the rapid disruption of cell walls, sonication facilitates the exhaustive extraction of bioactive components, which might subsequently modulate gene expression.

Medicinal plants can inhibit tumorigenesis by triggering apoptosis and regulating pathways such as the cell cycle. Their phytochemicals, present in different concentrations, contribute to anti-proliferative, anti-migratory, colony-inhibitory, and gene-modulating effects that may help prevent GC. Knocking down specific genes with shRNAs and inspecting these processes with and without extracts could elucidate their roles.

A potential limitation of our study is the lack of *in vivo* data, which could be attributed to the unavailability of experimental animals and the time constraints encountered during this study. Also, there were no validations in other GC cell lines. However, this limitation may pave the way for future research.

V. Conclusion

We concluded that crude methanol and acetone extracts of *C. azarolus* fruits and leaves could significantly exhibit potential anticancer capabilities against the AGS cell line compared to the normal fibroblast cell line. The AF and ML contained the highest amount of bioactive components. This plant's extracts also have anti-clonogenic properties, significantly reducing AGS cell line holoclone production, which is known to play a role in cancer progression. However, the exact mechanism remains unclear, but the induction of apoptosis could be one of the mechanisms for reducing cancer cell proliferation, and the upregulation of PTEN and NDRG1 interferes with the functional oncogenic role of EGR1 and/or EZH2 in inhibiting the apoptosis mechanism in AGS cells. The induction of apoptosis could block the oncogenic role of EGR1 and EZH2 in GC. These findings suggest that *C. azarolus* extracts may serve as a potential source of bioactive compounds for further investigation in GC treatment, either as an alternative to chemotherapies or in combination with them. As far as we are aware, this is the first report on the use of *C. azarolus* extracts in the treatment of GC cell lines.

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