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In vitro characterization of some of the anti-cancer effects of *Borage officinalis* flower extracts on human gastric adenocarcinoma cell line, (AGS), cell line

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ABSTRACT

Worldwide, gastric cancer is considered to be a major health issue, which is common in eastern Asia, with high death rates recorded, that calls for immediate attention and investigation. This article is exploring the effects of methanol and acetone extracts of borage, *Borage officinals,* flowers with a range of concentrations (10, 50, 100, 250, and 500 µg/ml) on the human gastric cancer, AGS cell line. Fifteen gm of flower powder was suspended separately in 300ml of 50% acetone and placed on shaker in dark for 24 hours at room temperature. The results showed that in a dose dependent manner, methanol extract exhibit anti-proliferative effects whereas acetone extract revealed an inhibitory effect only at a higher dose 500 µg/ml. The clonogenicity of AGS cells was revealed to be significantly ($p<0.001$) diminished where production of holoclone was reduced where cells treated with both extracts as compared to control group. AGS cells apoptosis levels were induced by both extracts when compared to control group. RT-PCR results depicted significant ($p<0.001$) upregulation of both *p21* and *EGR1* when cells treated with both methanol and acetone extracts respectively, while both of *NDRG1* and *PTEN* genes were upregulated when acetone extract was used, it's worth mentioning, that these genes, *p21*, *PTEN*, and *NDRG1* are considered to be tumor suppressors. In conclusion, borage, *Borage officinals*, extracts had anti-cancer and cytotoxic effects on AGS cell line and apoptosis was induced. Thus, this plant might be suggested as a future candidate for cancer treatment therapy.

1.Introduction

Cancer is an aberrant tissue growth in which the cells demonstrate uncontrollable cell division in an independent manner, which causes the number of the dividing cells to steadily rise (Hanahan, 2022). Gastric cancer (GC) is considered the fourth most recurrent cancer in the globe and the fifth most prevalent cancer overall, genetic and epigenetic factors affect the incidence of GC (Chela H et al., 2024). Given the fact that treatment of GC and other types of cancer, as such long term and inflammatory diseases are difficult, due to the diverse side effects which come along with the treatment processes, that they affect the ambient normal cells and tissues, in addition that these drugs are costly and not available in all countries (Yatoo et al., 2018). Thus, various medicinal plants such as borage, hawthorn, cloves and many other plants, have been employed to be used as substitutional drugs, tending to target different treating mechanisms both *in vivo* and *in vitro.* Apoptosis activation is the mechanism of choice by most candidate drugs for uncovering the potential effectiveness especially in cancer trials. In GC, there are many genes which play pivotal roles in GC progression for instance, the transcription activator, early growth response 1, (EGR1), it could be dysregulated in various cancer types where it might have a double function either a tumor suppressor or an oncogene depending on the type of cancer (Mohamad et al., 2018). The EZH2 on the other hand, is a member of polycomb repressive complex 2 (PRC2), regulate transcription of many genes that have role in differentiation, cell cycle progression, and stem cell plasticity. A strong connection has been demonstrated between modulation of tumorigeneses and PRC 2 activities in mediating H3K27me3 and gene silencing (Flora et al., 2021). Loss of PTEN protein functions have been recorded in many GC cases (Wang et al., 2021a).

Borage, *Borage officinals,* is an annual Mediterranean herb commonly known as starflower, it has been known for its medicinal use since long time ago (Ramezani et al., 2020). Historically, eatable flowers have been typically known for their use aroma and aesthetic appeal, but, nowadays world has started uncovering creative natural sources of bioactive substances within the flowers (Nowicka and Wojdylo, 2019). There are different bioactive secondary metabolites found in different parts of the plants (Mohammedsaeed and Mohamad, 2023) including edible flowers (Nowicka and Wojdylo, 2019). Natural bioactive compounds consumption and in accurate doses might be of advantages in preventing, controlling or treating various acute and chronic disorders including cancers (Fernandes et al., 2019). It is now well acknowledged that phytochemicals, such as antioxidants, are essential for recruiting up free radicals and preventing fatal diseases involving cancer, stroke, and heart-related illnesses (Hale et al., 2008), medicinal plants are well known for their antimicrobial effects as well (Khidhr et al., 2023).

The most significant phytochemicals in this regard are determined to be the phenolic and flavonoid compounds (Karimi et al., 2015). Furthermore, flowers are known for their carotenoid contents that can be applied in food preparation, pharmaceutical, and cosmetic industries. The carotenoids are considered as precursors of various vitamins in addition that they exhibit anti-inflammatory properties with immunomodulatory, antioxidant, and anti-cancer activities (Cardoso et al., 2017).

Numerous studies using a variety of gene types have shown that several important regulatory genes are involved in the initiation and development of various malignancies. Whereas the carcinogenesis may be linked to aberrant gene expression to various processes and targeting of various signaling pathways.

Therefore, this study was directed in order to investigate the anti-cancer activities of the bioactive components of *B. officinals* flower extracts involving, anti-proliferative, apoptotic, and anti-migration activities on gastric cancer cell line, AGS, as very little is known about the application of this flower extracts on gastric cancer.

2. Materials and methods:

2.1 Plant Collection

Borage officinalis flowers, was purchased from local market in Erbil city and the flowers were

dried in dark and stored at a non-humid place. This plant grows during November to January and could reaches a height of 70 to 100 cm. The plant was identified and authenticated by taxonomist, Mrs. Bnar K. Shkar, Lecturer at Biology department, College of Science, Salahaddin University, Erbil, Kurdistan Region-Iraq. The plant flowers were examined using organoleptic and macroscopic methods. Flower parts such as shape and colors were compared and matched to Herbarium sample, then it was confirmed by matching the other plant parts. Methanol and acetone dried flower extracts were prepared as described previously by (Mohammedsaeed and Mohamad, 2023).

2.2 Phytochemical Screening

Phytochemical screenings are considered as identification methods for plant constituents. Once the extraction was done, qualitative phytochemical screenings were done to locate different compound classes within the extracts of *B. officinalis* flowers. This screening is a group of chemical methods used for the detection of chemical compounds produced by plants, where different classes of compounds respond differently to color tests based on their solubilities and reaction with the different types of reagents for each tests and this color changes were detected by naked eye (Harborne, 1984). Table (1) shows the chemical reaction screening of number of plant compounds.

2.3 Cell Lines and Culture Conditions

Human gastric adenocarcinoma cell line, (AGS), was obtained from Pasture Institute, Iran. Medium F-12 (Sigma-USA) was used to culture the cells, the F-12 was complemented with 10% fetal bovine

serum (FBS), and (100 U/mL penicillin, 100 U/mL streptomycin; Gibco 15140122, Gibco, Waltham, MA, USA). Cells were cultivated at 37ºC in a moistened incubator with 5% CO2. The culture medium was changed every 2–3 days. For the sub-culturing and passaging, cells were washed with 1% PBS and trypsinized (0.25% trypsin EDTA; Gibco 25200056, Gibco,Waltham, MA, USA) for 60 seconds.

2.4 Cell Viability Assay

The effect of different concentrations of *B. officinalis* flower extracts on AGS cells proliferation (anti-proliferative effects) was performed by MTT assay. The principle of the assay dependents on the tetrazolium salt reduction by interacting with NADPH-dependent enzyme which is produced only by living cells, to produce purple insoluble formazan. Number of viable cells is taken from the absorbance of the solubilized formazan (Mustapha et al., 2015).

Growth inhibition was detected using MTT assay (Sigma Aldrich, USA). Human gastric adenocarcinoma cells, (AGS), a 96 well plate was used for seeding the cells at $10*10^3$ per well, incubated for 24 h at 37ºC, 5% CO2. Then different range concentrations of *B. officinalis* extracts (10, 50, 100, 250, and 500 µg/ml) were used for treating cells. After 48 h medium was discarded, then 100 µl of MTT reagent was added to each well, and cells were incubated for another 2-4 h. Followed by discarding medium and 100 µl DMSO 0.1% was added to wells. Cell viability was calculated at 540 nm by microplate ELISA reader (DA3200, Iran). Then cell counting was performed using hemocytometer in order to see the effects of the extract and which concentration might have the highest inhibitory effect.

2.5 Colony Formation Assay

A ~ 80% confluent of viable cells were treated with IC_{50} concentration (the dose required to inhibit cell growth by 50 percentage) of *B. officinalis* flower extracts. IC_{50} values were calculated using linear regression by plotting x-y and fit the data with a straight line, data were transformed logarithmically, then IC_{50} values were estimated using the equation below from the fitted line.

$Y=a*X+b$, $IC_{50} =(0.5 – b)/a$

IC_{50} values were (250 and 400 µg/ml for both methanolic and acetone extracts respectively) and untreated control, for two days. Trypsin was added in order to detach cells and harvest them and then seeded at ~ 2×10^2 cells per well of a 6-well plate and permitted to mature for fourteen days. Next, the cells were rinsed by PBS and incubated with 0.5% crystal violet solution for 30 min. Then the dye was removed with running tap water and plates were left to dry at room temperature (Wang et al., 2021b). The image was taken via Olympus inverted microscope 10X.

Colonies were counted by ImageJ software by opening an Image file, then from the tool bar many selections could be done like measuring, drawing, labelling and etc. The area selection tools allow to select the required area with a frame then analyzing could be done. Colonies counting was done using the measuring and counting objects by selecting the colonies then setting the measurements and start counting.

Table 1: Phytochemical Screening procedures to detect some chemical compositions in *Borage. officinalis* flower extracts.

	Chemical composition	**Reagent**	**Reagent preparation**	**Test Reaction**	**Expected positive result**
1	Alkaloids	Wagner	1.30gm of iodine and 2.0gm of potassium iodide in 30ml DW. then the volume was completed to 100ml.	Few drops of reagent into 1ml extract	Brown red precipitate
	(Harborne, 1984, Tyagi, 2017)				
2	Polyphenol (Tannins)	Lead Acetate Solution	1gm lead acetate into 100ml DW.	1ml of reagent with 1ml extract	White jelly precipitate
	(Harborne, 1984)				
3	Polyphenol (Flavonoids)	Sulphuric Acid	Sulphuric acid (H_2S0_4)	1ml reagent with 1ml extract	Yellow color
	(Velavan, 2015)				
4	Triterpenoids	Liebermann-Burchard	4ml of acetic anhydride with 1ml concentrated H_2S0_4	Few drop of reagent to 5ml extract	Blue-green color appear
	(Harborne, 1984)				
5	Peptides and free amino group	1% Ninhydrine	0.5g ninhydrine into 50ml DW.	1ml reagent to 1ml extract	Blue or violet color
	(Harborne, 1984, Velavan, 2015)				
6	Carbohydrate	Molish	1% alpha-naphthol and ethanol	1ml reagent to 1ml extract and then add few drops of concentrated sulfuric acid	Violet ring
	(Velavan, 2015)				
7	Glycosides	Benedict	Benedict (CuSO4 and NaOH)	1m reagent with 1ml extract	Color change or orange precipitate appear
	(Al-Shahaat, 1986)				
8	Protein	Biuret	1.5gm (CuSO4.5H2O) and 6gm of Sodium potassium tartrate in 500ml of DW. 375ml of 2 molar Sodium hydroxide. Mix both solutions in final volume 1000ml DW.	1ml reagent with 5ml extract	Violet color
	(Tyagi, 2017, Tyagi Phd, 2017)				
9	Saponins	-	-	Agitation	Formation foam above the liquid surface
	(Lakache et al., 2016)				

2.6 Quantitative real time PCR (qPCR)

The RNA extraction was performed by means of Trizol (Life Technologies, Carlsbad, CA). Nano-drop was used to assess the RNA quantity. DNase (Promega, Madison, WI) was added to treat the extracted RNA and from the total RNA, 2 µg, was reverse transcribed with MultiScribe MuLV reverse transcriptase. 40 ng cDNA was utilized for quantifiable polymerase chain reaction (qPCR) amplification with SYBR green PCR master mix (Amplicon RealQPlus 2x, High ROXTM, Denmark). Negative regulators were free from RT samples where no reverse transcriptase was added. Three un-related RNA samples were assessed for each time point in triplicate using quantitative RT-qPCR. RT-qPCR data were calculated using the comparative Ct method. Standard deviations from the mean of the [Δ] Ct values were measured. Table (2) contains the primer sequences which were used in this study. All mRNA values were normalized to that of beta ACTINE. Normalization was also confirmed by comparing samples against 18S. The RT-qPCR cycles were as follow: initial denaturation 95ºC for 5 min, denaturation step 95ºC for 30sce, annealing 60ºC for 20sec, extension step 72ºC for 20sec (40 cycles) and final extension 72ºC for 5 min.

2.7 Flow cytometric analysis of cell apoptosis

Treated AGS cells with one-time IC50 concentrations (250 and 400 µg/ml for both methanolic and acetone extracts respectively) of the flower extracts were evaluated for cell death rate by measuring the apoptosis or necrosis rate. Then adherent cells were trypsinized for 2 minutes and neutralized with FBS, then harvested and centrifuged (12000rpm, 25° C, 5 min). cells were suspended with 500µl 1x Annexin V Binding Buffer (Thermo fisher No=V13242). The cells then were stained with 10µl mixture of both propidium iodide 5µl (PI) and annexin V-fluorescein isothiocyanate (FITC) using an Annexin V-FITC and left at room temperature in dark for fifteen minutes. Cells were then examined using FACSCalibur Flow cytometer (Becton Dickinson, USA).

2.8 Statistical Analysis

Each test was done in triplicate for three un-related biological repeats. All data were analyzed by ANOVA, with the Brown-Forsythe test and GraphPad Prism8.0.2 (San Diego, USA) being expressed as mean ±SD. Data are presented as the arithmetic means ±SD of three independent experiments. The statistical significance of results obtained with probability values $P<0.05$, <0.01, and <0.001, being counted as significant.

3. Results and Discussion:

3.1 Phytochemical Screening of B. officinalis Flower Extracts

Phytochemical screening results of *B. officinalis* flower extracts Table (3), showed that there are number of the significant bioactive components such as tannins, flavonoid, carbohydrates and other components where the details of the results are in Table (3), each of these substances has its own bioactive characteristics. Different chemical composition could be found in the flower of the studied plant (Fernandes et al., 2019). It could be demonstrated that tannins, flavonoid, carbohydrates, and glycosides were found in highest quantities in both extracts, whereas, peptides was high in methanol but in lower quantity in acetone extract. The high contents of different phytochemical compounds within the borage extracts might be due to the solvent polarities which made it possible to dissolve and extract more ingredients (Zadernowskia et al., 2002).

3.2 Anti-proliferative Effect of B. officinalis Flower Methanolic and Acetone Extracts on AGS cell line.

Growth inhibition effects of both plant extracts were detected using the MTT assay. The results revealed that both methanol and acetone extracts of *B. officinalis* flowers revealed significant inhibitory (cytotoxic) effects on AGS cell line proliferation in a dose dependent manner (Figure 1). A 250 µg/ml concentration showed significant ($p < 0.001$) inhibitory effects on AGS cells, then interestingly with increasing dose (500 µg/ml), number of viable AGS cells significantly ($p<0.01$ and $p<0.001$) decreased relative to untreated control cells. While concentrations (10, 50 and 100 µg/ml) had no significant effects on cell viability. In regard to acetone flower extract, only the (500 µg/ml) showed significant inhibitory effect on the AGS cells viability. It has been

reported that methanol extract of borage flower had strong cytotoxic effects against number of cancer cells such as liver, HPG2, prostate, LNCaP, and colon, HT29, cancer cell lines. This anticancer function of methanolic extract might be assigned to the high phenolic and flavonoid contents (Karimi et al., 2017, Lozano-Baena et al., 2016). which might indicate the cytotoxic activities of this plant's components.

Table 2: Sequence of primers used for Quantitative Real Time PCR (qRT-PCR).

Genes	Primers		Ref.
EGR1	Forward	5' TTCCTCAGCTGTCACCAACTC 3'	Cinacolon company
	Reverse	5' CTCTGAGAACCTCCATCTGACC 3'	
EZH2	Forward	5' GACCTCTGTCTTACTTGTGGAGC 3'	
	Reverse	5' CGTCAGATGGTGCCAGCAATAG 3'	
NDRG1	Forward	5' TGGTTCCAGCGTCACTTCTC 3'	
	Reverse	5' ACCGAGTTAGGCGCAGTATG 3'	
PTEN	Forward	5' ACCAGGACCAGAGGAAACCT 3'	
	Reverse	5' GCTAGCCTCTGGATTTGACG 3'	
p21	Forward	5' CCAGCATGACAGATTTCTACCAC 3'	
	Reverse	5' GGCCAGGGTATGTACATGAGGAG 3'	
BETA-ACTIN	Forward	5' CAGGTGGTCTCCTCTGACTTCAAC 3'	
	Reverse	5' AGGGTCTCTCTCTTCCTCTTGTGC 3'	

Table 3: Phytochemical screening profile for borage, *B. officinalis* flower extracts.

No.	Chemical Components	Borage	
		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	+	+

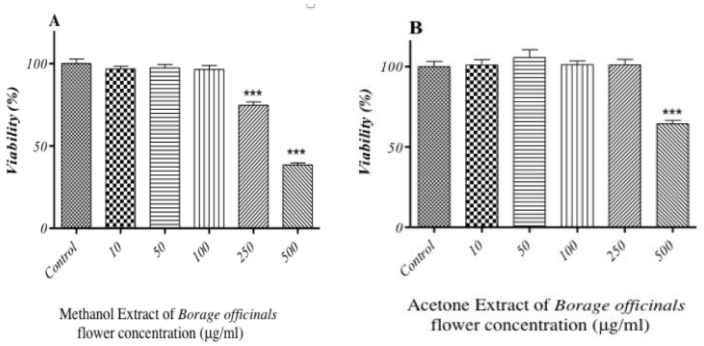

Figure 1: Proliferation inhibitory effect of borage, *B. officinalis* on AGS cell line. A) methanol flower extract and B) acetone flower extracts. Cells were treated with a range of extracts concentrations after 48h relative to untreated control; measured by MTT assay. Methanol extract showed a significant dose dependent manner in proliferation inhibitory effects, panel A. In case of acetone, panel B, only the highest concentration (500 µg/ml) revealed a significant inhibitory effects. The obtained values are presented as mean ±SD for at least three independent replications each with three replications (*= p<0.05, **= p<0.01, and ***= p<0.001).

3.3 Colony Formation Assay

Colony formation assay is also known as clonogenic assay, is performed in vitro to determine the capacity of a single cell to grow into a colony (Franken et al., 2006). The AGS cells' ability to form colony treated with *B. officinalis* extracts for two weeks, were assessed. Here, the distinguished capabilities of AGS cells were diminished to a significant point after being treated with the IC_{50} concentrations (250 and 400 µg/ml for both methanolic and acetone extracts respectively). Our results displayed that there were high significant differences ($p < 0.001$) between the treatments in regard to clonnumbers and their types (Figure 2). Methanol extract exhibited a reduction in holoclone number when compared to control group however it was not significant, also we demonstrated an increase in paraclone type when compared to control group (Figure 2A, and 2C).

On the other hand, acetone flower extract displayed higher significant ($p < 0.001$) reduction in type and number of the clones regarding to both holoclones and meroclones, while an increase in the paraclone type was recorded compared to all untreated control group (Figure 2A, and 2C). As it is well known that cancer cells tend to grow in colonies that come into contact with neighboring cells (Hanahan, 2022). When cancer cells lose contact with their neighbors, they die. The current study's results showed that all extracts effectively inhibited one of the essential traits needed by cancer cells to continuously grow and proliferate: their capacity to grow, proliferate, and form colonies, which subsequently helps to establish two crucial cancer hallmarks: migration and metastasis. The holoclones which are recognized for having the highest generative capacity and as densely organized cell bundles, spherical in shape (Flynn et al., 2020). This type is considered the most risky type of clones as they have the capabilities to self-renewal and proliferate extensively (Beaver et al., 2014), which means that they are more prone to metastasis and induce cancer in another location. While meroclones possess fixed proliferative abilities in addition that they are in-capable of self-renewal and that the least dangerous is the paraclones, where they have no ability for further division (Beaver et al., 2014). It has been found by another study that both numbers and types of holoclones and meroclones formed by AGS cells could be reduced by treating cells with methanol and acetone extracts of *Crataegus azarolus* (Mohammedsaeed, 2023). When compared to other clone types, holoclones have been reported to demonstrate substantial chemoresistance, which may pose a challenge to cancer cells' responses to chemotherapeutic medications. This could suggest that cells with paraclones may take up more chemicals than those with holoclones (Tan et al., 2011).

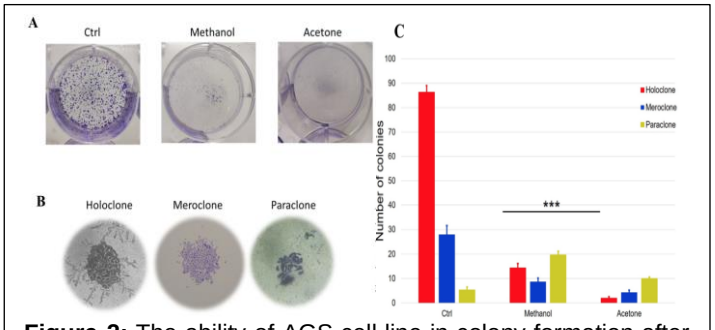

Figure 2: The ability of AGS cell line in colony formation after treatment with borage methanol and acetone extracts. A) AGS cell line colony formation after treatment with methanol and acetone borage flower extracts. B) Clone types formed by the treated AGS cells. C) The colonies number of each type formed by treated AGS and untreated control cells. The values are depicted as mean ±SD for at least three independent replications each with three replications (***= p<0.001).

3.4 Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Different types of genes were studied in several studies, and have demonstrated that there are many key regulatory genes which have engaged in the development and progression of different cancers. Where abnormal expressions of different genes might be attributed to the carcinogenesis approaching different mechanisms and targeting several signaling pathways. According to many studies it has been demonstrated that upregulation of *EGR1* and *EZH2* have role in GC progression (Ma et al., 2021). *PTEN, p21,* and *NDRG1* are known for their tumor suppressor characteristics, in addition to their role in cell cycle regulation (Mohamad et al., 2018). The results of gene expression levels result are presented in (Figure 3). Target genes expression were obtained and normalized with beta-Actin, the reference gene/housekeeping gene. AGS cells treated with methanol and acetone extracts indicated that *p21* and *EGR1* were upregulated significantly, while acetone extract had effects in upregulating both of *NDRG1* and *PTEN* genes. Whereas *EZH2* interestingly did not show any significant changes when compared to untreated cells. Many studies have reported that upregulation of both *EGR1* and *EZH2* play a pivotal role in GC progression. *EGR1* plays a significant and positive part in GC, where it has been elucidated that it is correlated with inhibiting GC from being progressed as low expression of EGR1 was related with the progression of some types of cancers (Feng et al., 2022, Michalak et al., 2023, Mohamad et al., 2018).

It has been recorded that *p21* higher levels induced apoptosis (Feng et al., 2022). The upregulation of *NDRG1*, *PTEN*, and *p21* together in our study might suggest driving cells to apoptosis given that extracts of other plants could induce apoptosis in AGS cells (Zou and Chang, 2011), and hence these genes are associated to apoptosis pathways and inhibiting proliferation, thus reducing GC prognosis, especially when *EZH2* didn't reveal any change in its expression which might refer to that the activation of apoptosis pathway. The tumor suppressor genes *PTEN* and *NDRG1* were considerably up-regulated in the examined plant extracts which could interfere with the role of the oncogenes, *EGR1* and *EZH2*, in inhibiting apoptosis mechanism in AGS cells. However, the un-changed expression of *EZH2* might indicate another apoptosis pathway independent of *EZH2*.

3.5 Flow Cytometry (Apoptosis)

Flow cytometry was used to evaluate rate of apoptosis for the treated AGS cells. The results which are displayed in (Figure 4) show that both methanol and acetone flower extracts induced apoptosis program in different degree. The percentage of both early and late apoptosis for methanol extract treatment was (65.67%) when compared to untreated control (50.45%), on the other hand we demonstrated a high significant induction of apoptosis when cells treated with acetone extract (145.9%) comparing to control group. The results revealed that when cells were treated with the extracts, there were reduction in the percentage of live cells and it was significant ($p < 0.001$) with acetone. Intriguingly, it was demonstrated that acetone had high significant ($p < 0.001$) in triggering both early and late apoptosis when compared to control and methanol. Whereas, methanol extract was significant ($p < 0.001$) in inducing only early apoptosis comparing to un-treated cells. This effect might be attributed to flower's components like flavonoids and phenolic compounds on enhancing programed cell death of AGS cells. The induction of apoptosis aids in the translocation of the mitochondrial voltage-dependent anion channel membrane and mitochondrial disturbance, which drive the discharge of cytochrome c and other pro-apoptotic proteins such as BCL-Xs. The function of this pro-apoptotic is derived from its capacity to disrupt the BAK/VDAC complex through its interaction with voltage-dependent anion channel (VDAC), thus freeing BAK for activation into cytoplasm which leads to caspase activity (Warren et al., 2019). Zou and Chang demonstrated that extract from black soybean could induce apoptosis in AGS cells (Zou and Chang, 2011). In another study, the induction of intrinsic apoptosis pathway was also reported when different concentrations of anthocyanins

were used for AGS cells treatment. An elevation in the levels of caspase-3 and pro-apoptotic proteins, Bax, were also detected, caspase-3 activation was resulted from the accumulation of cells at G0/G1 phase of cell cycle. This lead to proteolysis of PARP, in turn cell cycle arrest and sequentially induces apoptosis (Shih et al., 2005).

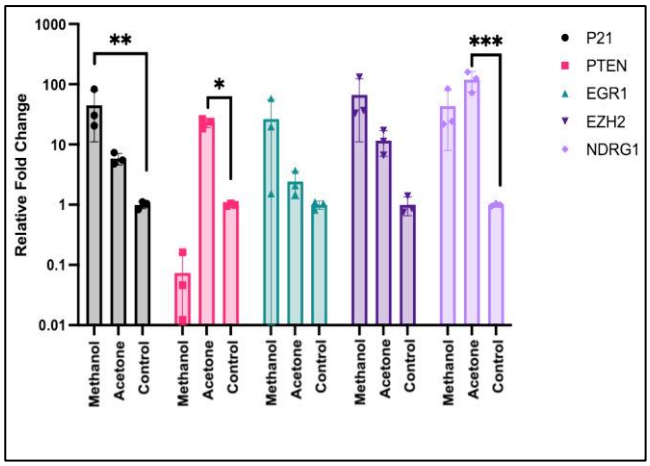

Figure 3: Transcriptional profile of genes *p21, PTEN, EGR1, EZH2* and *NDRG1* in AGS cell line after treatment with IC50 extracts compared to control. Beta-Actin as the reference gene and the relative expression of target genes were normalized with *Beta-Actin*. *p21* and *EGR1* were upregulated when treated with both methanol and acetone extracts, while acetone extract had effects on upregulating both of *PTEN and NDRG1..* All expression values of all studied genes were compared to untreated control. (**= p<0.01, and ***= p<0.001).

4. Conclusion

In this current investigation, it's been concluded that AGS cell line proliferation might be significantly inhibited when treated with either methanolic or acetone crude extracts of borage. The AGS cells had a prominent clonogenic property. *B. officinalis* extracts significantly decreased the holoclone and the viability of AGS cells by inducing apoptosis and modulating cell cycle. Furthermore, the extracts exhibited attractive anti-cancer, cytotoxic effects. It is also possible to draw the conclusion that increased expression of the tumor suppressor genes *PTEN* and *NDRG1* may conflict with *EGR1's* and/or extracts in GC cell line treatment. *EZH2's* functional oncogenic involvement in preventing AGS cells from going through the apoptotic process induction. Interestingly, it could be concluded that the obtained results might be attributed to the different bioactive components. To the best of our knowledge, this study could be reported as the first study in using *B. officinalis* extracts in GC cell line treatment.

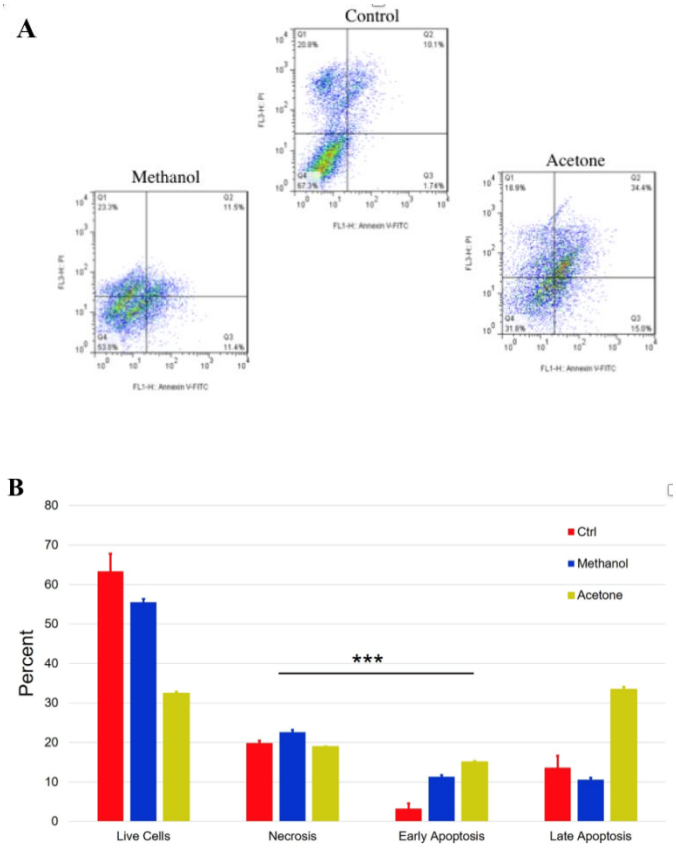

Figure 4: Apoptotic levels of AGS cell line treated with extracts using flow cytometry. A) AGS cell line scatter graph, Q1 (necrosis), Q2 (late apoptosis), Q3 (early apoptosis), and Q4 (live cells). This graph is one representative scatter graph out of three independent experiments. B) Statistical representation of apoptotic cells after treatment in comparison to untreated AGS cells together with cells treated with the methanol and acetone flower extracts respectively. The obtained values are presented as mean ±SD for at least three independent replications each with three replications (***= p<0.001).

Conflicts of Interests

The authors declare that they have no conflict of interests.

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