Cytotoxic Activity of Salvia Officinalis Extract Mediated by the Inhibition of JAK2 in Breast Cancer

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Cytotoxic activity of *Salvia Officinalis* extract mediated by the inhibition of JAK2 in breast cancer

A THESIS SUBMITTED BY

Salma Alawadi Dawood

TO THE

Biotechnology graduate program

*In partial fulfillment of the requirements for the degree of*

*Master of Sciences in Biotechnology*

Supervised by

*Dr. Ahmed Abdellatif*

Assistant professor, Department of Biology

Spring 2023
Declaration of Authorship

I, Salma Alawadi Dawood, declare that this thesis titled, “Cytotoxic activity of Salvia Officinalis extract mediated by the inhibition of JAK2 in breast cancer” and the work presented in it are my own.

I confirm that:

• This work was done wholly or mainly while in candidature for a research degree at this University.

• Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.

• Where I have consulted the published work of others, this is always clearly attributed.

• Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.

• I have acknowledged all main sources of help.

• Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed:

Date:
Cytotoxic activity of *Salvia Officinalis* extract mediated by the inhibition of JAK2 in breast cancer

**Abstract**

**Background:** Breast cancer is the most common type of invasive cancer in women in their forties and fifties. Recent evidence suggests that JAK2/STAT3 signaling is constitutively active in breast cancer. Previous studies suggest that plant extracts, including *Salvia Officinalis*, have strong cytotoxic effects on breast cancer cells. The differential expression of miRNAs is also strongly linked to cancer development.

**Aim:** In the current study, we hypothesize that *S. Officinalis* extract suppresses JAK2 expression and has strong anticancer potential in MCF7 breast cancer cell lines.

**Methods:** GC-MS analysis showed the presence of flavonoids in dried leaf of *Salvia officinalis* Extract. The cytotoxicity of *S. Officinalis* was compared to Cisplatin on human breast (MCF-7) cells. Bioinformatic analysis was performed to detect the link between JAK2 and different microRNAs. qPCR assessment of microRNAs and JAK2 , BAX, Bcl-xL and BIRC5 mRNAs was performed. miR-216a-5p was overexpressed in MCF7 cells to test its anticancer potential.

**Results:** GC-MS analysis showed the presence of anticancer and antioxidant compounds (Linolein and Apigenin). *S. Officinalis* extract reduced cell proliferation of MCF-7 cells with an IC50 range from 5.123 to 6.345 mg/mL (p<0.0001) compared to cisplatin (IC50 =20 ug/ul). *S. Officinalis* was also safe for the human skin fibroblast, suggesting that *S. Officinalis* has anticancer activity and is less harmful to normal cells (p<0.0001). Morphological assessment of MCF-7 cells showed that untreated cells maintained their epithelial morphological shape, while those treated with *S. Officinalis* displayed morphological changes consistent with apoptosis. Bioinformatic analysis revealed that JAK2 contains theoretical binding sites of miR-101, miR-216, and miR-204 in its 3’ UTR. qPCR revealed that three miRs (miR-101-5p, miR-216a-5p & miR-204-5p) were less expressed in breast cancer cell lines than in normal cell lines (P=0.0022). *S. Officinalis* and cisplatin reduced the expression of miR-101-5p (p<0.0001). While *S. Officinalis* reduced the expression of miR-216a-5p (p<0.005).
Also, qPCR showed that *S. Officinalis* and miR-216a-5p mimics significantly reduced JAK2 mRNA expression (p<0.0001). *S. Officinalis* and overexpression of miR-216a-5p both increased BAX expression while decreasing Bcl-xL and BIRC5 expression.

**Conclusions:**

*S. Officinalis* has significant anticancer potential mediated through the increased mRNA expression of BAX and reduced expression of JAK2, Bcl-xL, and BIRC5 mRNAs. As well as the reduced expression of miR-101-5p & miR-216a-5p. Although the overexpression of miR-216a-5p in MCF7 increased BAX expression and significantly decreased JAK2, BIRC5, and Bcl-xL expression, it did not lead to cell death *in vitro*.

**Keywords:** Salvia Officinalis, miR-101-5p, miR-216a-5p & miR-204-5p, JAK2, BAX, Bcl-xL, BIRC5
Dedication

With a heart full of love and gratitude, I would like to dedicate this work
To my life’s role model, my father, and my beloved mother, for their continuous
love and support.

To my dear brothers, Ahmed & Sherif,

and To my precious friends, Ashrakat, Marwa & Omnia.

& To all breast cancer fighters.
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Conclusion and future perspectives

References
**List of Abbreviations**

- AKT: Ak strain transforming.
- BAX: Bcl-2 Associated X-protein
- Bcl-xL: B-cell lymphoma-extra large
- BIRC5: Baculoviral inhibitor of apoptosis repeat containing 5
- BRCA 1 & 2: Breast Cancer Type 1 and 2
- cDNA: Complementary DNA (Deoxyribonucleic acid)
- C-met: C-mesenchymal-epithelial transition factor
- C-myc: Cellular myelocytomatosis oncogene
- Dapi: (4′,6-diamidino-2-phenylindole)
- DTT: Dithiothreitol
- DGCR8: DiGeorge syndrome chromosomal region 8
- dNTP: Deoxynucleoside triphosphates
- DMEM: Dulbecco’s modified Eagle’s medium
- DMSO: Dimethyl sulfoxide
- DRV: DNA readouts viewers
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- GC/MS: Gas Chromatography/Mass Spectroscopy
- HeLa: Henrietta Lacks
- HEK: Human embryonic kidney cell lines
- HSF: Human Skin Fibroblast cell lines
- HER2: human epidermal growth factor receptor 2
- TG-5MS: TraceGOLD-5 mass spectroscopy
- EI: Electron impact
- LNCaP: Lymph Node Carcinoma of the Prostate
- MCF-7: Michigan Cancer Foundation -7
- McI-1: Myeloid cell leukemia sequence-1
- IL-6 & 10: Interleukin 6 and 10
- FBS: Fetal Bovine Serum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog deleted on Chromosome 10</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase and PI3 kinase.</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NIH</td>
<td>National Library of medicine</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous carcinoma cancer cell lines</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>Oligo(dT)</td>
<td>Oligonucleotides(deoxythymidine)</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless/Integrated</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Runt-related transcription factor 1</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>UTRs</td>
<td>Untranslated regions</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SNORD</td>
<td>C/D-box snoRNAs</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sex determining region Y-box 2</td>
</tr>
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*S. Officinalis*  *Salvia Officinalis*
Chapter 1 Introduction

1.1 Breast cancer.

Breast cancer is the most common invasive cancer type in middle-aged and older women, accounting for 38.8% of cancers in Egypt, with a death rate of nearly 11% (Ibrahim et al. 2014). Breast cancer risk is caused by a combination of factors. Although most women have some risk factors, the majority of women do not develop breast cancer. The majority of breast cancers are found in women over the age of 50. Furthermore, women with a family history and those with inherited mutations in certain genes, such as Breast Cancer Type 1 and 2 (BRCA1 & 2), are more likely to develop breast and ovarian cancer (Shiovitz and Korde 2015).

Breast cancer is treated in a variety of ways, including surgery, chemotherapy, radiation, targeted and hormonal therapy, all of which have side effects. Pain and lymphedema can result from surgery. Hot flashes, joint pain, and bone thinning can all be side effects of hormonal therapy. Itching, soreness, and peeling skin can all be side effects of radiation therapy. Chemotherapy and targeted therapies can have similar side effects, such as vomiting, fatigue, and diarrhea, hair loss, neuropathy, and mouth sores (Peart 2015; Maughan, Lutterbie, and Ham 2010). Despite all types of treatments, breast cancer remains a leading cause of cancer death. Therefore, many studies are focusing on better understanding the cellular mechanisms that lead to tumor formation and cancer metastasis in hopes of achieving higher cure rates

1.2 JAK2/STAT3 pathway

Signal transducers and activators of transcription (STATs) are transcription factors involved in a variety of biological processes, including proliferation, apoptosis, and metastasis (Siveen et al. 2014). STAT3 overactivation is associated with breast cancer progression, metastasis, and chemoresistance (Siveen et al. 2014). STAT3 protein can be activated by cytokines like interleukin 6 and 10 (IL-6 & IL-10), as well as other growth factors (Ma, Qin, and Li 2020). It is also used as a tumor diagnostic marker.
Janus kinases (JAKs) are associated with the cytoplasmic domains of cytokines receptors to activate STAT3 (Siveen et al. 2014). When cytokine receptors such as IL-6 are activated, JAK2 tyrosine kinases are phosphorylated and activated, allowing the phosphorylation and activation of cytoplasmic STAT3 protein (Ma, Qin, and Li 2020). The phosphorylated STAT3 forms homodimers and translocates into the nucleus to form a complex with some coactivators. This binds to the promoter region of target genes to activate transcription of genes involved in proliferation and apoptosis, such as Cyclin D, c-myc, Bcl-2, and Bax, survivin (Y. Wang et al. 2018). An outline of the JAK2/STAT3 pathway is shown in Figure 1.1.

![Cytokine Signaling through the JAK2/STAT3 Pathway](image)

**Figure 1.1 The JAK2/STAT3 pathway.**

Recent evidence suggests that JAK2 signaling is linked to certain types of cancer as it influences proliferation and apoptosis, as demonstrated by abnormal STAT3 signaling in malignant cells (Sen et al. 2015). In addition, JAK2/STAT3 signaling is constitutively active in breast cancer cells (Garcia et al. 2001). Furthermore, JAK2 gene mutations, as well as environmental, clinical,
and hematological risk factors, are associated with breast cancer (Karim et al. 2019). JAK2 (valine-to-phenylalanine) mutation is one of the most common mutations (Karim et al. 2019). Inhibiting the JAK2/STAT3 pathway reduces cell viability, invasion, and migration and induces cell apoptosis (M. S. Kim et al. 2015).

The JAK2/STAT3 pathway has been the target of many research strategies to identify therapeutics for breast cancer. Licochalcone (LC), for example, significantly inhibited the JAK2/STAT3 pathway, resulting in the downregulation of target genes such as Bcl-2, survivin, cyclin D1, p21, and p27 (also known as cyclin-dependent kinase inhibitor). Thus, resulting in the reduction of cell growth and apoptosis (Oh et al. 2019). Ruxolitinib is a selective inhibitor of JAK1/2, which inhibits STAT3 activation and decreases cell growth in breast cancer (Lim et al. 2018), non-small lung cancer (Taverna et al. 2020), hepatocellular carcinoma (Gu et al. 2016), cervical cancer (Morgan and Macdonald 2019), and colorectal cancer (Gu et al. 2016).

However, JAK inhibitors suppress the immune system and increase susceptibility to serious infections (Papp et al. 2021). In addition, they increase the risk of serious heart or blood vessel problems and certain types of cancer, such as skin cancer (Papp et al. 2021). Another JAK2 inhibitor is Fedratinib which was approved for myelofibrosis and other solid tumors. However, it may cause encephalopathy, drowsiness, difficulty understanding or speaking, loss of memory, and vision problems (Talpaz and Kiladjian 2021).

1.3. Role of *Salvia Officinalis* in Breast Cancer.

Because of the serious complications of currently available drugs, the search for a safe and cheap alternative is ongoing. *Salvia officinalis* (Sage) is a plant in the Lamiaceae family. It originated in the Middle East and the Mediterranean areas (Gorbani and Esmaeilizadeh 2017). Salvia has been used as a herbal medicine to treat a variety of diseases, such as inflammation, age-related cognitive disorders, and cancer (Gorbani and Esmaeilizadeh 2017). It also has anticancer and antioxidant properties (Jiang, Zhang, and Rupasinghe 2016). The main bioactive ingredients in *Salvia officinalis* are alkaloids, carbohydrates, fatty acids, glycosidic derivatives, phenolic compounds (coumarins, flavonoids, tannins), steroids, and terpenes (Jiang, Zhang, and Rupasinghe 2016). *Salvia miltiorrhiza* (Red Sage) and related species were also found to have
antiproliferative activity in human breast cancer MCF-7 by blocking cell entry into the S phase, decreasing AKT phosphorylation, and increasing P27, a cell cycle inhibitor (Jiang, Zhang, and Rupasinghe 2016). Salvia sahendica derivatives inhibited cell proliferation by upregulating PTEN and activating caspase-3 to induce apoptosis in breast cancer cells (Esmaeili and Farimani 2014).

Salvia officinalis extracts contain bioactive compounds such as carnosol, luteolin, and cirsiliol which inhibit STAT3. Carnosol and Cirsiliol reduce the DNA binding of STAT3 and affect the phosphorylation of Janus kinase (JAK2) and Src kinase (Yanagimichi et al. 2021). On the other hand, luteolin promotes the degradation of phosphorylated STAT3 in liver cancer cells (Selvendiran et al. 2006).

1.4. MicroRNAs in Breast Cancer

Micro-RNAs are non-protein-coding RNAs with a length of about 20 nucleotides that regulate a wide range of genes involved in many cellular processes such as cell cycle, apoptosis and cell proliferation (Si et al. 2019; Shah et al. 2016). Many studies have demonstrated the effects of microRNAs on a variety of diseases, particularly cancer. MiRNAs may act as oncogenes and tumor suppressors (Shah et al. 2016). microRNAs such as miR-21, miR-210, and miR-221 were significantly upregulated; however, miR-10b, miR-145, miR-205, and miR-122 were significantly downregulated in cancers of the breast, colorectal and lymphoma, as it affects proliferation, apoptosis, and migration of cancer cells in vitro (C.-Z. Wang et al. 2018; Singh and Mo 2013). The overexpression of miR-143 suppressed some proteins that are involved in breast cancer cell viability, such as mitogen-activated protein kinase (MAP3K7) and cyclin D1 (Zhou et al. 2017). miR-101 was also reported to affect cancer cell proliferation, apoptosis, angiogenesis, drug resistance, invasion, and metastasis in human triple breast cancer (Xiaoping Liu et al. 2015). Another study suggested that miR-101 regulates JAK2 expression and affects cervical cancer cell proliferation and apoptosis. (L. Wang et al. 2014).
Moreover, the miRNA-related genes also served as key players in the growth, proliferation, cycle, and apoptosis of cells (Shah et al. 2016). MiR 216a-5p has been shown to act as a tumor suppressor in breast cancer (Esquela-Kerscher and Slack 2006). It also regulated autophagy in human umbilical vein endothelial cells by regulating Beclin1 expression (Menghini et al. 2014). MiR-216a-5p was also suggested to have a tumor suppressor role that inhibited the malignant effects (Li and Ma 2015). Recent studies suggested that miR-216a-5p regulates the expression of some genes, such as Nanog, SOX2 and OCT4, and JAK2 (Lee et al. 2022; Roscigno et al. 2020). Other studies suggested a link between miR-216a and the JAK2/STAT3 signaling pathway. By targeting JAK2, miR-216a negatively regulated the development of pancreatic cancer cells (S. Wang, Chen, and Tang 2014; Lee et al. 2022). Thus, it may be used to inhibit cell proliferation and induce cell apoptosis.

The miRNAs' differential expression is strongly linked to the initiation, progression, apoptosis, and metastasis of breast cancer (Iorio et al. 2005; Singh and Mo 2013). Down-regulation of tumor-suppressing miRNAs, on the other hand, may result in increased oncogene function and increased expression of target genes, promoting tumor formation (Shah et al. 2016). Consequently, targeting microRNA can be used as a potential therapeutic strategy either alone or in combination with other therapeutics.

This study was performed to investigate the mechanism of action of *S. Officinalis* and its link to the JAK2/STAT3 pathway and its possible interaction with JAK2, BIRC5, Bcl-xL mRNAs, as well as other microRNAs.

**Hypothesis**

*S. Officinalis* suppresses JAK2 expression and exerts strong anticancer potential in MCF-7 breast cancer cells.

**Aims of the work**

1- Investigate the cytotoxic effect of *S. Officinalis* on MCF-7 breast cancer cells.
2- Investigate the role of *S. Officinalis* on the expression of JAK2, BAX, BIRC5 & Bcl-xL at mRNA levels.
3- Investigate the relationship between JAK2 and other microRNAs.
Figure 1.2 The hypothesized mechanism of action of *Salvia officinalis*.

*Salvia officinalis* plant extract is hypothesized to have anticancer activity by targeting JAK2/STAT3 pathway.
Chapter 2 Material and Methods

2.1 Gas Chromatography-Mass Spectroscopy (GC-MS)

*Salvia officinalis* Dried leaves extract (333 mg/ml, Hawaii Pharm, HI, USA) was analyzed using GC-MS mass spectrometer (Thermo Scientific, Austin, TX, USA). The components were identified by comparing their retention times and mass spectra to the mass spectral databases WILEY 09 and NIST14 (El-Kareem et al. 2016).

2.2 Cell viability (Cytotoxicity) assay

Breast cancer cells lines MCF-7 (Michigan Cancer Foundation – 7) were cultured in T75 flasks containing Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Belgium) in addition to 10% Fetal Bovine Serum (FBS, Life Science group, U.K.), and 1% Penicillin-Streptomycin (Lonza, Belgium). Cultured cells were incubated in humidified 95% air, and 5% CO2 at 37°C. Cells were detached using 0.2% (w/v) trypsin and centrifuged and resuspended in 5 mL of cell culture medium in a T25 flask and incubated for 24 hrs before running the experiments. MCF-7 cells were grown to approximately 80-90% confluency before each experiment was performed.

Human Skin Fibroblast (HSF) cell line was also used to test the level of cytotoxicity of *Salvia Officinalis* on normal cell lines. Cisplatin 50mg/50ml (India, Mylan) was also tested on MCF-7 cell lines as a positive control. Eight concentrations of cisplatin were evaluated: (160, 80, 40, 20, 10, 5, 2.5, 1.25 µg / ml of cisplatin). Graph Pad Prism 9 was used to calculate the IC50.

Plant extract (100 µl) diluted with media was added to the cells. *Salvia Officinalis* extract was tested at eight different concentrations (160, 80, 40, 20, 10, 5, 2.5, and 1.25 mg/mL). Three different experiments were performed in triplicates.

The effects of *Salvia Officinalis* were evaluated after 24, 48, and 72 hours. The cell viability of the MCF-7 cell line was evaluated by the standard MTT (3,4,5-dimethyl-thiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT; Serva Electrophoresis, Germany) reduction assay for 3 hr. After carefully aspirating the supernatants, 120 µl of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to each well to dissolve the precipitate dark blue formazan crystals. The absorbance of formazan was measured at 570 nm using a microplate reader (SPECTROstar Nano
microplate reader, BMG Labtech) at 38°C, which is directly proportional to the number of live cells.

Cytotoxicity assay was also used to assess MCF7 cell viability after transfection with miR-216a-5p mimics. The following equation was used to compute viability (% of cell viability) 

\[ V = \frac{A_t}{A_c} \times 100 \]

Where "At" represents the absorbance of treated cells, and "Ac " represents the absorbance of control cells. Graph Pad prism 9 was used to determine the IC50.

2.3 Microscopic examination and cell counting

Three different concentrations (5, 10, and 20 mg/mL) were chosen for further experiments. Dapi staining was used to determine morphological and nuclear changes. MCF-7 breast cancer cells were treated for 24 hours with Salvia Officinalis in 96 well culture plates. The cells were then fixed by adding 100 µl of methanol for 10 minutes, followed by washing with 100 µl of PBS. A 100 µl of DAPI working solution was added to each well for 20 minutes, followed by washing with PBS. Then, the pictures were captured by using a fluorescent microscope at two different magnifications.

The Trypan Blue exclusion method was used for cell counting. Cells were rinsed with PBS and detached with 0.2% trypsin after 24 hours of treatment. Ten microliters (µl) of this cell suspension were mixed with ten microliters (µl) of Trypn blue, and viable cells were counted using the Neubauer hemocytometer chamber. The number of cells per 1 mL of cell suspension was calculated using the following equation:

\[ \text{Number of cells/ml} = \frac{\text{Total numbers of viable cells in all counted squares}}{\text{total number of counted squares}} \times \text{dilution factor x10000.} \]

2.4 RNA extraction

Breast cancer cells (MCF7, 30 x 10^4) were treated with 5 mg/mL Salvia Officinalis in 6 well-plates for 24 hr. After incubation, the culture media was removed, and cells were trypsinized, centrifuged, and lysed with 1 ml of Triple Xtractor (GRISP). Chloroform (200 µl)
was added, and cells were centrifuged at 12000 g at 2-8 °C for 15 min. This resulted in the separation of RNA in the aqueous phase; the aqueous solution was transferred to a new Eppendorf, and isopropanol was added (500 μl per 1 ml of Triple Xtractor), then centrifuged again at 12,000g for 10 min.

RNA pellets were precipitated and washed twice with 70% ethanol. The tube was centrifuged at 12.000g for 5 min. The supernatant was discarded and left to air dry for 15 to 20 min to remove trace amounts of ethanol. The RNA pellet was dissolved in 30 μl of RNase-free water and incubated at 65 °C for 10 min.

RNA was also extracted from Human Skin Fibroblast (HSF) and Human Embryonic kidney cells (HEK) to investigate baseline expression of JAK2 and miRNAs in breast and normal cells.

The concentration of total RNA in the samples was determined using a NanoDrop 2000 (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA), ( OD260/280= 1.8-2). Agarose gel electrophoresis was performed to ensure the integrity of RNA yield. We observed that 28S and 18S or ribosomal RNA bands were clearly identified.

cDNA Synthesis

For reverse transcription, a GScript First-Strand Synthesis Kit (GeneDireX, Inc) was used. The protocol was followed according to the manufacturer's instructions to obtain cDNA. In brief, 5 μl of total RNA samples were mixed with 4 μl of 5X 1st strand buffer, 1 μl Oligo(dT)20, 1 μl DTT, 1 μl dNTP mix, 2 μl GScript RTase, and 6 μl RNase free water in a PCR strip and run in a thermocycler (BioRad, USA) programmed at 50°C, 60 minutes; 95°C, 15 min and hold at 4°C. Immediately the synthesized cDNA was stored at −20°C.

2.5 Prediction of miRNAs

To identify potential interactions with JAK2, bioinformatic analysis was performed using five databases (TargetScan (https://www.targetscan.org/vert_80/), miRanda (https://bioweb.pasteur.fr/packages/pack@miRanda@3.3a), miRDB (https://mirdb.org/), miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php))
and Diana Tools ([www.microrna.gr](http://www.microrna.gr)) to obtain a list of potential hsa-miR-216a-5p, miR-204-5p, and 101-3p downstream target genes.

**miRTarBase** and **DIANA** tools were used to obtain experimentally verified miRNA-target interactions (MTIs) using SNPs and DRVs in miRNAs and gene 3’UTRs, as well as miRNA expression levels across extracellular vesicles, blood, and different organs, to discover miRNA functions and identify potential targets of interest for downstream studies and wet lab activities. (John et al. 2004). **miRanda** addresses both binding site and position conservation, as well as matching the whole miRNA sequence (John et al. 2004).

**TargetScan** ranks projected targets based on either predicted targeting efficacy (context+ scores) or the likelihood of conserved targeting (PCT). The conservation of a 3’ UTR is determined first, followed by a study of a specific k-mer (8mer, 7mer-m8, or 7mer-1A). An aggregate PCT is supplied since a single 3’ UTR can contain numerous target sites. For each type of k-mer, the number for that target, as well as whether it is considered a conserved or poorly conserved site, is provided (Friedman et al. 2009). The context+ score indicates how likely it is that a specific target will be successfully targeted. **miRDB** was used to get target prediction for mature miRNAs.

A short list of common genes was identified. Some of these genes are **FOXC1, SOX2, Caspase 3, RUNX1, mTOR, and JAK2**, which are essential for cell growth, proliferation, and maintaining self-renewal and apoptosis (Sood, Kamikubo, and Liu 2017; Persad et al. 2004; Graves and Milovanova 2019; Levine et al. 2007; Zou et al. 2020).

The JAK2 gene was selected for further assessment in the current study due to its potential role in cancer development. We also identified potential interactions between miR-101, miR-216 & miR-204, and putative binding sites in the JAK2 3’-UTR predicted by Target Scan and other bioinformatics tools.

### 2.6 Quantitative Polymerase Chain Reaction (q-PCR)

The baseline expression of JAK2 in normal cells (HSF and HEK) and breast (MCF-7) cells was determined, and the effects of *Salvia officinalis* (5mg/mL), miR-216a-5p mimics (25 nm), and
cisplatin (20 μg/μl) on the selected genes were investigated. Table 1 lists all primers used for amplification of the selected genes. HERA plus SYBR green qPCR kit (WF1030800X, Willowfort, UK) was used according to the manufacturer’s instructions. In brief, 2 μL of cDNA mixed with 10 μL of 5x Syber green master mix, 2 μL of forward primer, 2 μL of reverse primer, and 4 μL RNase free water were mixed. The following PCR cycles were performed: 95°C for 10 minutes, 40 cycles of 1 minute at 95°C, and 30 seconds at 60°C. The melting curve was estimated at the end of the runs, and the data was analyzed using the comparative threshold cycle (Ct) method. Normalization was accomplished in comparison to the mean of housekeeping genes. (GAPDH and β-ACTIN).

Quantitative RT-PCR was also performed to determine miR-101-5p, miR-216a-5p, and miR-204-5p baseline levels by using miRCURY® LNA® miRNA PCR Assays (Qiagen) in (MCF-7) breast cancer cell lines and HSF (Human skin fibroblast). In addition, the expression level of miRs was tested after using both S. officinalis (5mg/ml) and cisplatin (20 μg/μl) on MCF-7 cells.

cDNA was diluted 1:40 by adding 390 μl RNase-free water. Table 2 lists the primers used for amplification of the selected genes. The miRCURY® LNA® miRNA SYBER Green PCR kit (Qiagen) was used as instructed by the manufacturer. 10 μL total reaction volume was prepared: 1 μL of cDNA mixed with 5 μL of 2x miRCURY SYBR Green master mix, 0.1 μL of Rox reference dye, 1 μL of miR primer, and 2.9 μL RNase free water was added to the mixture in the PCR strip mixture, and the reaction was run in an Applied biosystem 7500. The melting curve was estimated at the end of the runs and data were analyzed using the comparative threshold cycle (ΔΔCt) method. Normalization was achieved relative to the mean of housekeeping genes (SNORD).

2.7 Transfection of miRNA 216a-5p mimics

Cells (2 x 10*5) were reverse transfected with 25 nM of miRNA (hsa-miR-216a-5p mimics) or Negative control, using 3 μl of HiPerfect reagent according to the manufacturer's protocol. 5 μl of miR-216a-5p mimics stock solution (10 μM) was mixed with low serum opti-MEM media (250 μl) and HiPerfect reagent (3 μl). The mix was left at room temperature for 20 minutes to
allow the formation of the miRNA-Lipid complex. Cells (2 x 10^5) were suspended in 1.5 ml low serum opti-MEM media (Gibco), and the mix was added to 6 well plates. The serum-free media was replaced after 5-6 hours with antibiotic-free, complete DMEM media containing 10% FBS.

All primers used for the transfection are listed in Table 3. The negative control was subjected to the same procedures. Cells were either left untransfected (Untreated) or treated only with Hiperfect reagent as additional controls (Mock transfection). Cells were incubated for 24 hours after transfection before being harvested for RNA extraction. RNA extraction was performed as mentioned above.
Table 1. Primers used for selected genes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence of Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH (Human)</strong></td>
<td>Forward: 5’GCACAGTCAAGGCCGAGAAT 3’&lt;br&gt;Reverse: 5’GCCTTCTCCATGGTGTTGAA 3’</td>
</tr>
<tr>
<td>NM_001256799.3</td>
<td></td>
</tr>
<tr>
<td><strong>JAK2 (Human) NM_001322194.2</strong></td>
<td>Forward: 5’-CCGCTCGAGAAGAAATGACCTTCATTCTG AGATTA-3’&lt;br&gt;Reverse: 5’-AAGGAAAAAAAGCGGCCGCTAAAGTAAGAA ACTATTTTCTTTTTAATCAAAAC3-’</td>
</tr>
<tr>
<td>Main target: ENST00000381652, Chromosome Location: Chr.9: 4985245 - 5128183</td>
<td></td>
</tr>
<tr>
<td><strong>BCL-2 (Human) NM_001317919.2</strong></td>
<td>Forward: 5’CAGAGCTTTGAACAGGTAG 3’&lt;br&gt;Reverse: 5’GCTCTCGGGTGCTGTATTG 3’</td>
</tr>
<tr>
<td><strong>BAX (Human) NM_001291430.2</strong></td>
<td>Forward: 5’GGTTTGCGCCCTTTTTCTA 3’&lt;br&gt;Reverse: 5’CGGAGGAAGTCCAATGTC 3’</td>
</tr>
<tr>
<td><strong>BIRC5 or Survivin</strong></td>
<td>Forward: 5’ AGTGAGGGAGGAAGAAGGCA 3’&lt;br&gt;Reverse: 5’ ATTCACTGTGGAAGGCTCTGC 3’</td>
</tr>
<tr>
<td>NM_001012270.2 Homo sapiens baculoviral IAP repeat containing 5 (BIRC5), transcript variant 2, mRNA</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2: Primers used for MicroRNAs.

<table>
<thead>
<tr>
<th>MiRNA</th>
<th>Mature Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-216a-5p</td>
<td>5<code> UAAUCUCAGCUGGCAACUGUGA 3</code></td>
</tr>
<tr>
<td>miRBaseAccession: MI0000739</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-204a-5p</td>
<td>5<code> UUCUUUGUCAUCCAUUGCCU 3</code></td>
</tr>
<tr>
<td>miRBase Accession: MI0000284</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-101-5p</td>
<td>5<code> UCGGUUAUCAUGGUACCGAUGC3</code></td>
</tr>
<tr>
<td>miRBase Accession: MI0000739</td>
<td></td>
</tr>
<tr>
<td>hsa-SNORD38B</td>
<td>5<code>UCUCAGUGAUGAAAACUUUGUCC3</code></td>
</tr>
<tr>
<td>miRBase Accession: SNORD38B</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3: primers used for miRNA mimics

<table>
<thead>
<tr>
<th>miRNAs mimics</th>
<th>Mature sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-216a-5p mimics</td>
<td>5<code> UAAUCUCAGCUGGCAACUGUGA 3</code></td>
</tr>
<tr>
<td>hsa-miRNA mimics Negative control 4</td>
<td>5<code> GAUGGCAUUCGAUCAGUUCUA 3</code></td>
</tr>
</tbody>
</table>

### 2.8 Statistical analysis

Data were analyzed using GraphPad Prism v.9 Software Inc. (San Diego, CA, USA). Data were expressed as the mean ± standard deviation (SD). P-values < 0.05 were considered significant. One-way ANOVA test was followed by Dunnett's test, and Two-way ANOVA was followed by Tukey's for multiple comparisons.
Chapter 3 Results

3.1 Chemical composition of *S. Officinalis*

GC-MS mass spectrometer (Thermo Scientific, Austin, TX, USA) was used to analyze the chemical composition of the extract dried leaves of *Salvia officinalis* (333 mg/ml, Hawaii Pharm, HI, USA).

There were huge similarities between the compounds that were found in aqueous and ethanolic extracts. Five compounds were detected in both the aqueous and ethanolic extracts. The most abundant compound was Linolein (42.45%). The second compound that was commonly found in *S. Officinalis* is Apigenin (27.23%). Other compounds were found in small percentages, such as Chamazulene (0.29%) and Spathulenol (2.51%). In addition, the presence of glycerol (25.14%) was used to prepare the alcohol-free liquid extract. (Table 4).

According to the literature review and PubChem (NIH), we found that all four compounds have anti-inflammatory activity. Apigenin and Linolein have anticancer activity. In addition, both have a role in antioxidants. Linolein also has a role in cardiovascular diseases, and Apigenin plays a role in neuroprotective diseases. These findings highlight the anticancer activity of both compounds linolein and apigenin in plant extract of *Salvia Officinalis*. 
Table 4. The chemical composition of *Salvia Officinalis* extract.

<table>
<thead>
<tr>
<th>(Retention Time RT) Compound Name</th>
<th>Area %</th>
<th>Molecular Formula and Structure *</th>
<th>Mol. Weight</th>
<th>Pharmacological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(18.94) Spathulenol</td>
<td>2.51</td>
<td><img src="image" alt="Chemical structure of Spathulenol" /></td>
<td>220</td>
<td>Antioxidant, anti-inflammatory, antiproliferative (do Nascimento et al. 2018).</td>
</tr>
<tr>
<td>(43.12) linolein</td>
<td>42.45</td>
<td><img src="image" alt="Chemical structure of linolein" /></td>
<td>878</td>
<td>1-Anti-inflammatory (Bakun et al. 2021; Ramadan et al. 2006)(Luo, Shang, and Li 2017)) 2- Anticancer (Fauziah et al. 2022). 3- Antioxidant and Cardiovascular diseases (Luo, Shang, and Li 2017).</td>
</tr>
<tr>
<td>(39.49) Chamazulene</td>
<td>0.29</td>
<td><img src="image" alt="Chemical structure of Chamazulene" /></td>
<td>184</td>
<td>1- Anti-inflammatory (Bakun et al. 2021; Ramadan et al. 2006). 2- Antineoplastic, anti-diabetes, antiretroviral, and antimicrobial (Bakun et al. 2021).</td>
</tr>
</tbody>
</table>
Table 4 shows the chemical composition of both ethanol and water extract of *Salvia Officinalis*, which were analyzed by GC-MS. The active principles of retention time (RT), percentage of peak area (Area %), molecular formula (MF) and structure, molecular weight (MW), and pharmacological activity of each compound are presented in the table 4.

### 3.2 Cytotoxicity of *Salvia Officinalis*

The cytotoxicity of *S. officinalis* was assessed using concentrations ranging from 1.25 to 160 mg/mL on the MCF-7 cell line after 24, 48, and 72 hours of exposure. At higher concentrations of 160 mg/mL, the extract was toxic to the MCF-7 cell line, causing 20 to 30% cell viability after 24, 48 & 72 hours of exposure (Figure 3.1). However, at lower concentrations (1.25 and 2.5 mg/mL), cell viability was greater than 90% after 24, 48, and 72 hours of exposure. (Figure 3.1).
In general, cell viability reduced as plant extract concentrations increased. At the concentrations (5 and 10 mg/ml), cell viability started to decline by 50 to 60% after 24, 48, and 72 hours of exposure (P<0.0001, Figure 3.1).

The IC50 of S. officinalis extracts on the MCF-7 cell line was calculated after 24, 48, and 72 hours of exposure (Table 5). The lowest concentration of plant extract that inhibits 50% of cells (IC50 value) was obtained using Graph Pad Prism 9. The IC50 ranged from 5.460 to 6.635 mg/mL after 24 hr of exposure.

### 3.3. Cytotoxicity of Cisplatin on MCF-7 cell line.

Cisplatin (cis-diamine-dichloro platinum II) is a metal-based anti-cancer drug commonly used in breast cancer. Cisplatin was used as a positive control in the current study. MTT assay was used to assess the cytotoxicity of cisplatin at concentrations ranging from 1.25 to 160 ug/ml on the MCF-7 cell line. The effect of cisplatin on the MCF-7 cell line was studied after 24, 48, and 72 hours of exposure. At higher concentrations of 160 ug/ml, the drug was toxic to the MCF-7 cell line, causing 10% to 40% cell viability. At lower concentrations (1.25 and 2.5 ug/ml), cell viability ranged from 60% to 90% after 24, 48, and 72 hours of exposure. In general, cell viability reduced as cisplatin concentration increased (Figure 3.2). IC50 of cisplatin was obtained by Graph prism. According to these results, 20 ug/ml concentration caused 50 % inhibition of cell proliferation after 24 hr of exposure to the drug (P<0.0001, Figure 3.2). Therefore, this concentration (20 ug/ml) was used for further experiments.

We compared the effect of S. Officinalis and cisplatin on human breast cancer MCF-7 cell proliferation by MTT assay (Figure 3.3). The IC50 of S. Officinalis ranged from 5.460 to 6.472 mg/mL, from 5.168 to 6.486 mg/mL, and from 5.130 to 6.635 mg/mL, after a 24, 48, and 72 hr of treatment, respectively, compared to the IC50 value of cisplatin of 20, 10 and 5 ug/ml.
3.4. Cytotoxicity of Salvia Officinalis on Human skin fibroblast (HSF) cell line.

To assess the safety of Salvia Officinalis against normal cells, HSF cells were used. The cells were treated with the same eight concentrations that were performed on MCF-7 cell lines (1.25, 2.5, 5, 10, 20, 40, 80, 160 mg/mL) of S. Officinalis for 24 and 48 hours. No significant cytotoxic effect was observed on the normal HSF at the low concentration (1.25, 2.5, 5, and 10 mg/ml) (Figure 3.4). After 24 hours of treatment, Salvia officinalis concentrations ranging from 20 to 160 mg/ml began to reduce HSF cell proliferation. Cell proliferation, however, decreased rapidly from 5 to 80 mg/ml after 48 hours of therapy, reaching nearly 20% inhibition. This effect began to fade at 160 mg/ml (Figure 3.4).

At 24 hours and the IC50 concentration (5.5 to 6.6 mg/ml), it is clear that the extract is not toxic to the normal cells compared to its effect on the MCF-7 cell line (Figure 3.5). These findings suggested that the Salvia officinalis extract could generate equivalent anticancer activity with far fewer harmful effects on normal cells.

3.5. Salvia Officinalis induced apoptotic changes on MCF-7 Cells.

MCF-7 breast cancer cells were exposed to three different concentrations for 24 hrs of Salvia Officinalis (5, 10, and 20 mg/mL) and then examined under a microscope (Figure 3.6). Untreated cells maintained their normal epithelial shape. However, cells treated with Salvia Officinalis showed cell death and morphological changes that are typical of apoptosis, such as chromatin condensation. Cell death increased as S. Officinalis concentrations increased.

3.6. Trypan blue cell viability assay

At 24 hours of exposure to three concentrations of Salvia Officinalis (5, 10 & 20 mg/mL) remarkably decreased the number of live cells in MCF-7. Trypan blue exclusion assay (Figure 3.7) showed that the number of dead cells significantly increased (p-value = 0.0003) after 24 hr exposure to S. Officinalis in the MCF-7 cell lines.

These results, along with the data obtained by trypan blue exclusion, confirm the previous results. The 5 mg concentration was chosen for further experiments.
Figure 3.1. Cytotoxicity of *S. Officinalis* on MCF-7 cell lines after 24, 48, and 72 hours of exposure.

Extract concentrations ranging from 5 to 160 mg/mL showed a significant reduction in cell viability compared to untreated cells (P<0.0001, n=12). Lower concentrations (1.25 and 2.5 mg/mL) did not show cytotoxic effects.

Table 5. The IC50 of *S. Officinalis* extract on MCF-7 cells.

<table>
<thead>
<tr>
<th></th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>5.460 to 6.472 mg/mL</td>
<td>5.168 to 6.486 mg/mL</td>
<td>5.130 to 6.635 mg/mL</td>
</tr>
</tbody>
</table>
Figure 3.2. Cytotoxicity of cisplatin on MCF-7 cell lines after 24, 48, and 72 hours of exposure.

Cisplatin concentrations ranging from 1.25 to 160 ug/ml showed a significant reduction in cell viability after 24, 48, and 72 hours compared to untreated cells (P<0.0001, n=8). Cisplatin exposure for 48 and 72 hours also significantly reduced cell viability compared to 24 hours. IC50 of cisplatin was obtained by using Graph prism (20, 10, 5 ug/ml after 24, 48 and 72 hours of exposure).
A

Cytotoxicity of *S. Officinalis* and cisplatin on MCF-7 cell lines after 24 hr

```
% cell viability
```

Concentrations

```
Negative Control 160 80 40 20 10 5 2.5 1.25
```

- Salvia Officinalis (mg/ml)
- Cisplatin (μg/ml)

B

Cytotoxicity of *S. Officinalis* and cisplatin on MCF-7 cell lines after 48 hr

```
% cell viability
```

Concentrations

```
Negative Control 160 80 40 20 10 5 2.5 1.25
```

- Salvia Officinalis (mg/ml)
- Cisplatin (μg/ml)
Figures 3.3 Comparison of the cytotoxic effects of *S. Officinalis* and cisplatin on MCF-7 cell viability.

Figures show the comparison between *S. officinalis* and cisplatin cytotoxic effects after 24, 48, and 72 hrs of exposure using MTT assays. (A) shows cytotoxicity of *S. officinalis* and cisplatin after 24 hr incubation. (B) shows the results of the MTT assay after 48 hr of exposure. Figure C shows the effect on cell viability after 72 hr. *S. officinalis* was more effective at 24 hr compared to cisplatin. However, cisplatin caused lower cell viability at 48 and 72 hours (P<0.0001, n=8).
Figure 3.4 Cytotoxicity of *S. Officinalis* on HSF (Human Skin Fibroblast) cell line.

*S. Officinalis* extract is safe at concentrations ranging from 1.25 to 10 mg/mL after 24 hours of exposure. At concentrations ranging from 1.25 to 20 mg/mL *S. officinalis* did not cause significant cell death after 24 hours, while higher concentrations were toxic. Concentrations above 1.25 were cytotoxic at 48 hours, compared to untreated cells and 24 hr exposure (P<0.0001, n=8).
Figure 3.5. The effect of *S. Officinalis* on MCF-7 and HSF cell viability after 24 & 48 hr of exposure.

MCF7 and HSF cells were exposed to *S. officinalis* for 24 and 48 hrs. At concentrations ranging from 1.25 to 10 mg/ml, the extract did not show toxic effects on HSF cells at 24h, while at higher
concentrations, the extract was toxic. At 48 hr, the extract was toxic to both MCF7 and HSF cells at concentrations higher than 1.25 mg/mL (P<0.0001, n=8).

**Morphological assessment of MCF7 cells.**

<table>
<thead>
<tr>
<th>Negative Control</th>
<th>5 mg</th>
<th>10 mg</th>
<th>20 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>D</td>
<td>G</td>
<td>J</td>
</tr>
<tr>
<td><img src="image1.png" alt="Photomicrograph A" /></td>
<td><img src="image2.png" alt="Photomicrograph D" /></td>
<td><img src="image3.png" alt="Photomicrograph G" /></td>
<td><img src="image4.png" alt="Photomicrograph J" /></td>
</tr>
<tr>
<td>B</td>
<td>E</td>
<td>H</td>
<td>K</td>
</tr>
<tr>
<td><img src="image5.png" alt="Photomicrograph B" /></td>
<td><img src="image6.png" alt="Photomicrograph E" /></td>
<td><img src="image7.png" alt="Photomicrograph H" /></td>
<td><img src="image8.png" alt="Photomicrograph K" /></td>
</tr>
<tr>
<td>C</td>
<td>F</td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td><img src="image9.png" alt="Photomicrograph C" /></td>
<td><img src="image10.png" alt="Photomicrograph F" /></td>
<td><img src="image11.png" alt="Photomicrograph I" /></td>
<td><img src="image12.png" alt="Photomicrograph L" /></td>
</tr>
</tbody>
</table>

**Figure 3.6 Morphological Assessment of MCF7 cells treated with S. Officinalis.**

The photomicrographs show the effect of *Salvia Officinalis* extract (5, 10 & 20 mg/mL) on the MCF-7 cell morphology. Light microscope images of MCF-7 (A, D, G, J) and DAPI-stained cells (B, C, E, F, H, I, K, L). Untreated MCF-7 cells (A, B, C) maintained their normal epithelial MCF-7 morphology. However, cells treated with *S. Officinalis* (D - L) showed cell death and
morphological changes that are typical of apoptosis, such as chromatin condensation (red arrows, Mag 20x, and 40x, n=12).

**Figure 3.7 Trypan blue viability assay.**

The figure shows the number of viable cells following the treatment of MCF-7 cells for 24 hours. Three concentrations were used (5, 10, and 20 mg/ml). Untreated MCF-7 cells had an average of 4,500,000 viable cells. *Salvia Officinalis*, on the other hand, caused a significant decrease in viable cells to 1,130,000, 255,000, and 200,000 viable cells in cells treated with 5, 10, and 20 mg/mL, respectively (p=0.0003, n=8).
3.3 *Salvia Officinalis* targets JAK2/STAT3 Signaling Pathway.

In this study, we used qPCR to investigate the baseline expression of JAK2 in MCF-7, HEK, and HSF cells. We found that the baseline expression of JAK2 in MCF7 (Figure 3.8) was significantly upregulated compared to that of the HEK and HSF cell lines (Figure 3.8, **** P<0.0001, * P=0.031), which indicates that it is expressed in cancerous cells. Then, we investigated the effect of *Salvia Officinalis* and its relation to the JAK2/STAT3 pathway. qPCR assays revealed that *S. Officinalis* dramatically (P<0.0001) reduced the transcription of JAK2. In addition, cisplatin reduced JAK2 expression (Figure 3.8).

*S. Officinalis* and cisplatin also reduced survivin (BIRC5) mRNA levels (*** p< 0.001) while increasing the transcription of BAX mRNA (**** P<0.0001). Furthermore, *S. Officinalis* and cisplatin significantly reduced the transcription of Bcl-xL mRNA (**** P<0.0001) by qPCR (Figure 3.9). This suggested that targeting the JAK2 pathway reduces tumor growth and leads to the induction of apoptosis.

3.4 Effects of *Salvia officinalis* on the expression levels of miRNAs.

**Prediction of miRNA**

Bioinformatic analysis using five databases ([TargetScan](https://www.targetscan.org/vert_80/)), [miRanda](https://bioweb.pasteur.fr/packages/pack@miRanda@3.3a), [miRDB](https://mirdb.org/), [miRTarBase](https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php) and [Diana Tools](https://diana acompanied) predicted that JAK2 contained theoretical binding sites of miR-101-3p, miR-216a-5p, and miR-204-5p in its 3’ UTR (Figure 3.10 & Figure 3.8).

In this study, we used miR-101-5p after confirming that the sequence of miR-101-3p and miR-101-5p were identical. Further details are mentioned in the appendix.

qPCR was used to investigate the miRNAs (miR-101-5p, miR-216a-5p, and miR-204-5p) baseline expression in MCF-7 and HSF cell lines. The three miRs were found to be less
expressed in MCF-7 cell lines compared to HSF cell lines (Figure 3.11, *** p=0.005, ** p=0.001).

The effect of *Salvia Officinalis* on miRNA expression (miR-101-5p, miR-216-5p & miR-204a-5p) was compared to cisplatin. Cells were treated for 24 hours with 5 mg/mL (*S.Officinalis*), and cisplatin 20 ug/ul. qPCR assays revealed that both *S.Officinalis* and cisplatin significantly (P<0.0001) reduced the expression of miR-101-5p (Figure 3.12, **** p<0.0001) compared to untreated cells (Negative control). *S.Officinalis* also reduced the expression of miR-216a-5p (** P<0.005), while cisplatin had no significant effect on the expression level of miR-216a-5p (Figure 3.12). *S. Officinalis* and cisplatin, however, did not significantly change the miR-204-5p expression (Figure 3.12).
Figure 3.8 Expression of JAK2 in MCF7, HEK, and HSF cells.

The figure shows the baseline expression (A) of JAK2 in MCF7, HEK, and HSF cells. (B) Both *S. Officinalis* (5 mg/mL) and cisplatin (20 ug/ul) reduced the expression level of JAK2 significantly compared to untreated cells (**** P<0.0001, * p=0.031, n=8).
Gene expression in MCF7 cells treated with *S. Officinalis* and Cisplatin.

Figure 3.9. The effect of *Salvia Officinalis* and cisplatin on the expression of BAX, BIRC5 & Bcl-xL mRNAs.

The figure shows the effects of *S. Officinalis* (5 mg/mL) and cisplatin (20 μg/μl) on the expression level of BAX, BIRC5 & Bcl-xL mRNAs. *S. Officinalis* and Cisplatin upregulated the expression of Bax and downregulated the expression of BIRC5 & Bcl-xL. (** **P<0.0001. *** p< 0.001, * p=0.031, n=8).
**Figure 3.10.** The predicted binding sites of miR-101-3p, miR-216-5p & miR-204-5p in the 3′-UTR of JAK2.
Figure 3.11 Baseline expression of miR-101-5p, miR-216a-5p, and miR-204-5p in normal cells (HSF) and MCF7 cell lines.

All three miRNAs were significantly low expressed in MCF7 cells compared to Human skin fibroblast (HSF) cells (*** p=0.005, ** p=0.001, n=8).
Figure 3.12 Effects of *Salvia Officinalis* and cisplatin on the expression of miRNAs.

The figure shows the effect of *Salvia Officinalis* (5mg/ml) and cisplatin (20μg/μl) on the miR-101-5p, miR-216a-5p, and miR-204-5p expression in breast cancer MCF-7 cells. A significant drop in expression levels of miR-101-5p (**** p<0.0001, n=8). *Salvia Officinalis* (5mg/ml) reduced miR-216a-5p expression compared to cisplatin (20 μg/μl) (** P<0.005, n=8). However, miR-204-5p was not significantly different from untreated cells.
3.5 Overexpression of miR-216a-5p suppresses JAK2 expression

Cell viability assessment (MTT Assay) showed that cell viability was not affected by the transfection of miR216a-5p mimics after 24 and 48 hours of exposure. There was no significant difference between transfected, mock transfected, negative control, and untreated cells (Figure 3.13).

JAK2 expression was significantly upregulated in MCF7 cells than in normal cells (Figure 3.8). In our work, we found that the overexpression of miR-216a-5p in MCF7 cells significantly reduced the expression of JAK2 mRNA (Figure 3.14, **** p=0.0014). Treatment of MCF7 cells with *S. Officinalis* significantly reduced the expression level of JAK2 mRNA significantly compared to that following cisplatin treatment and after miR-216a-5p overexpression (Figure 3.14, ****p<0.0001). These results show that miR216a-5p may possibly have the potential to be used as a therapeutic tool for breast cancer.

Overexpression of miR-216a-5p in MCF7 changed the levels of BAX, BIRC5 & Bcl-xL mRNA (Figure 3.15). It increased the expression level of BAX (** p=0.003). On the other hand, it significantly reduced the expression level of BIRC5 and Bcl-xL (* p=0.01).
Figure 3.13. Cell viability assessment following cell transfection with miR-216a-5p.

The figure shows that cell viability was not affected by the transfection of miR-216a-5p (25 & 50 nm) after 24 and 48 hours of exposure. There was no significant difference between transfected, mock transfected, negative control, and untreated cells (n=10).
Figure 3.14. The expression of JAK2 after *S. Officinalis* (5 mg/mL), Cisplatin (20 μg/μl) effect, and miR-216a-5p mimics (25 nm).

The figure shows the JAK2 mRNA expression level in MCF7 cells (negative control), HSF, HEK cells, and the following treatment of MCF7 cells with Cisplatin, *S. Officinalis*, and overexpression of miR-216a-5p. In HSF cells, JAK2 mRNA was undetectable. MCF7 cells had significantly higher basal expression than HEK cells. Treatment of MCF7 cells with *S. Officinalis* significantly reduced the expression level of JAK2 mRNA significantly compared to that following cisplatin treatment and after miR-216a-5p overexpression. The overexpression of miR-216a-5p in MCF7 cells led to a significant reduction in the expression of JAK2 mRNA. (**** p<0.0001, ** p=0.0014, * p=0.01, n=6), (Negative control is untreated MCF-7 cells).
A. Effect of overexpression of miR216

B.

- Salvia Officinalis (5 mg/ml)
- Cisplatin 20 μg
- miR-216-5p mimics (25 nm)
Figure 3.15. The comparison between the effects of *S. Officinalis* (5mg), cisplatin (20μg) & miR-216a-5p mimics (25 nm) on the expression levels of BAX, BIRC5 & Bcl-xL mRNA.

Overexpression of miR-216a-5p, Cisplatin, and *S. Officinalis* treatment of MCF7 increased the expression level of BAX. On the other hand, BIRC5 and Bcl-xL were significantly reduced. miR-216a-5p, and Cisplatin significantly reduced BIRC5 compared to *S. Officinalis*. Cisplatin reduced expression Bcl-xl compared to *S. Officinalis* and miR-216a-5p mimics (* p=0.02, ** p=0.003, *** p= 0.001, **** p= 0.0001, n= 6).
Chapter 4 Discussion

Despite all types of treatments, breast cancer remains the second leading cause of cancer death in women (Peart 2015; Maughan, Lutterbie, and Ham 2010). Current treatment of breast cancer has side effects, ranging from pain and lymphedema to hot flashes and bone thinning (Peart 2015; Maughan, Lutterbie, and Ham 2010). Therefore, many studies are focusing on better understanding the cellular mechanisms that lead to tumor formation and cancer metastasis, in hopes of achieving higher cure rates.

In the current research, we investigated the safety and efficacy of *S. Officinalis* as well as its possible mechanism of action in breast cancer in MCF-7 cell lines.

MCF-7 cell lines were used in this study. This cell line was isolated from the breast tissue of a 69-year-old Caucasian woman in 1970. The first of her two mastectomies revealed that the removed tissue was benign. Five years later, a second operation revealed a malignant adenocarcinoma in a pleural effusion, from which tissue was extracted to create the MCF-7 cell line. Radiotherapy and hormone therapy were used to treat the donor's breast cancer. Furthermore, MCF-7 cell lines are the most common type of estrogen receptor-positive breast cancer. Approximately 80% of all breast cancers express the estrogen receptor (ER+) and are dependent on estrogen for cell growth and spread. (Vantangoli, M. M., 2015).

MCF-7, an extensively researched epithelial cancer cell line derived from breast adenocarcinoma, exhibits characteristics of differentiated mammary epithelium. These cells can process estradiol through cytoplasmic estrogen receptors. The presence of hormones in the serum required in tissue culture media makes demonstrating specific hormone and antihormone effects more difficult. The human breast cancer cell line MCF-7 is a limitless source of homogeneous self-replicating material that is free of contaminating stromal cells and can be easily cultured in standard media. MCF-7 human breast cancer cells were adapted to long-term growth in low serum concentration (0.5% foetal bovine serum) by gradually decreasing the medium serum content from 5% to 0.5% FBS. Tamoxifen is effective against MCF-7/S0.5 cells because they express both estrogen and progesterone receptors. (Vantangoli, M. M., 2015). However, other
studies used cisplatin as a positive control in MCF-7 compared to hypericin (Mirmalek et al. 2016). Therefore, we used cisplatin here as positive control as it was available in our laboratory.

**Chemical composition of S. Officinalis**

The current study shows that Linolein and Apigenin are key chemical compounds found in *S. Officinalis* in aqueous and ethanolic extract (Table 4). They are common dietary flavonoids abundantly present in a variety of fruits and vegetables, such as parsley, grapes, apples, and chamomile tea. These results are consistent with the current findings. For example, a study found that linolein and apigenin were the primary bioactive components in *S. Officinalis* that originated from Jordan (Dordevic, Cakic, and Amr 2000). Both Linolein and Apigenin have antioxidant properties (Fauziah et al. 2022; Salehi et al. 2019) and anticancer (Salehi et al. 2019). Apigenin also may have a role in neurodegenerative diseases (Yanagimichi et al. 2021). As evidenced by the inhibition of cell proliferation and induction of apoptosis in hepatocellular carcinoma (HepG2) and breast cancer (MCF-7) cell lines, including HER2-positive ones (Scherbakov and Andreeva 2015). Previous reports show that Apigenin demonstrated antitumor activity by modulating multiple signaling pathways such as PI3K/AKT, NF-kB, JAK/STATs, and Wnt/catenin (Yan et al. 2017). Knekt and colleagues also suggested a link between flavonoids like Linolein, Apigenin, Kaempferol, and Myricetin, and lung cancer (Knekt et al. 1997). They also discovered an inverse relationship between flavonoid consumption and cancer incidence across all study sites.

Other compounds found in small amounts in *S. Officinalis* included Chamazulene, which has anti-inflammatory, antineoplastic, antidiabetic, antiretroviral, and antimicrobial activity (Bakun et al. 2021; Ramadan et al. 2006).

To date, several studies have been conducted to assess the anticancer effects of *Salvia Officinalis*. For instance, a study showed that *S. Officinalis* has anticancer activity in three cell lines (MCF-7, HeLa, and LNCaP cell lines) by reducing cell growth and inducing apoptosis (Privitera et al. 2019). In light of these results and the data reported in the literature, these findings encouraged
further investigations on the mechanism of the cytotoxicity activity of *S. Officinalis* on MCF-7 breast cancer cells.

**Cell viability (Cytotoxicity) Assessment**

In the current study, *S. Officinalis* extract reduced the cell proliferation of breast cancer cells with IC50 values ranging from 5.460 to 6.472 mg/mL. Other studies reported some variability in the IC50 ranging from 100 μg/mL to 200 μg/mL on MCF-7, HeLa, and LNCaP cell lines after 48 hours of treatment (Privitera et al. 2019). Khiya et al. reported that *Salvia officinalis* collected from Morocco had an IC50 of 309.42 mg/ml (Khiya et al. 2019). Another study also showed that the IC50 of dichloromethane extract of *S. officinalis* was 8.95 ± 0.23 mg/mL in the diabetes mellitus *in vitro* model (Javid, Moein, and Moein 2021). This variability is possibly due to the nature of the crude extract that was used in the previous studies and the variability of the extraction methods and plant parts used.

Other studies found that *Salvia miltiorrhiza* alcohol/water extracts from Taiwan had strong antiproliferative activity on human oral squamous carcinoma (OSCC) cell lines, with IC50 values ranging from 39.8 to 47.1 μg/ml and 26.67 to 30.68 μg/mL were observed after 24 and 48 hours, respectively (W.-H. Wang et al. 2017). Furthermore, methanolic extract of the bark of the Libyan *Salvia fruticosa* inhibited the proliferation of three breast cancer cell lines (MCF-7, T47D, and MDA-MB-468) with IC50 values of 30, 21.2, and 37 μg/ml, respectively (Eltawaty et al. 2020).

Cisplatin has been widely used in the treatment of many cancers, including breast, testicular, ovarian, and small-cell lung cancer, over the last four decades (Bosch et al. 2010). Cisplatin has also been used in combination with other drugs, such as taxans and 5-fluorouracil (Florea and Büsselberg 2011). We decided to use Cisplatin as a positive control in the current study. Cisplatin reduced cell proliferation by 50% at a concentration of 20 ug/ml. Similar findings were reported by other studies (Mirmalek et al. 2016) examining the cytotoxicity of cisplatin in breast cancer cell lines.
Even though we did not study the effect of the combination between cisplatin and *S. Officinalis* on MCF-7 cell lines, other studies (Salehi et al. 2019) showed that *Salvia miltiorrhiza* enhanced sensitivity to cisplatin in A549/DDP lung cancer cells mainly through suppression of the c-met/AKT/mTOR signaling pathway (Salehi et al. 2019).

*S. Officinalis* showed a high safety margin when tested on Human skin fibroblast (as a model of a normal cell line). There were no significant effects on cell survival at doses below 20 mg/mL. Therefore, *Salvia officinalis* could generate equivalent anticancer activity with fewer harmful effects on normal cells. Other studies support our findings that normal cells were substantially less vulnerable to the cytotoxic action of *Salvia officinalis* extract (zare shahneh et al. 2013).

The morphological assessment showed that MCF-7 cells treated with *Salvia Officinalis* displayed morphological changes consistent with apoptosis. Although in the current study, no specific staining for apoptosis was used, the treated cells displayed characteristics of apoptotic cells, such as chromatin condensation. DAPI staining also revealed that *S. Officinalis* treated MCF7 cells revealed mostly mitotic morphology, indicating that the cells were arrested during mitosis. Previous reports show that *S. Officinalis* and *Salvia chorassanica* (Golshan et al. 2016) induced similar morphological changes, indicating apoptosis in breast cancer MCF7 and MDA-MB-231 cells (Brindisi et al. 2021).

**Salvia Officinalis exerts its anticancer effect by targeting JAK2/STAT3 pathway.**

Previous reports show that the JAK2/STAT3 pathway is essential in the development and survival of cancer cells (Lai and Johnson 2010). JAK2 is a non-receptor tyrosine kinase that plays a role in cancer cell proliferation, angiogenesis, immune evasion, and anti-apoptosis (Qian, Yao, and Si 2011). Previous research has linked JAK2 dysregulation, mutation, and amplification to the progression of cancer. According to some studies, JAK2 acts as an oncogene in breast cancer, and any dysregulation in the JAK2/STAT3 signaling pathway is particularly linked to breast cancer (Behera et al. 2010). According to one study, JAK2 is overexpressed in breast cancer tissue and cell lines (X. Wang et al. 2015). Activation JAK signaling pathway is observed
in human breast cancer (Behera et al. 2010). Therefore, inhibiting JAK2 activity prevents STAT3 nuclear translocation and tumorigenesis (Hedvat et al. 2009), which may be considered a novel strategy for developing anticancer agents.

Many studies were performed to target JAK2. For example, Licochalcone and Ruxolitinib inhibited the JAK2/STAT3 pathway and down-regulated genes like Bcl-2, survivin, cyclin D1, p21, and p27, reducing cell growth and inducing apoptosis (Oh et al. 2019)(Papp et al. 2021). Other studies found that Ruxolitinib inhibited STAT3 activation and decreased cell growth in breast cancer (Lim et al. 2018), non-small lung cancer (Taverna et al. 2020), cervical cancer (Morgan and Macdonald 2019), and colorectal cancer (Gu et al. 2016). However, JAK inhibitors suppress the immune system and increase vulnerability to serious infections (Papp et al. 2021).

In the current work, we show that *S. Officinalis* reduces the expression of JAK2 at the mRNA level by 95% in comparison to cisplatin. Previous studies found that *Salvia miltiorrhiza* inhibited JAK2 mRNA transcription in 5-FU-resistant Gastric cancer cell lines, therefore reducing cell proliferation (Wu et al. 2022). In another study, luteolin isolated from *S. Officinalis* extract inhibited JAK2 kinase in HepG2 cell lines (Abubaker et al. 2015). Cisplatin, on the other hand, reduced JAK2 gene transcription by 30%, similar to previous reports (C.-J. Li et al. 2023). Other studies, however, found that Cisplatin (5 ug/ul) treatment activated JAK2 in the ovarian cancer HEY cell line (Abubaker et al. 2015). These differences could possibly be due to the use of different cancer cell lines.

Other studies showed the effects on phosphorylation of JAK2. A study revealed that targeting JAK2/STAT3 pathway led to downregulating both JAK2 and p-JAK2 in MCF-7. (L. Wang et al. 2014) In the current work, we did not investigate the effect of *S. Officinalis* in the p-JAK2. However, we found that plant extract significantly reduced the JAK2 in MCF-7 cell lines.

The main targets of JAK2 activation are STAT3, Bax, Bcl-xL, and Survivin (BIRC5) (Ma, Qin, and Li 2020). In this study, we found that the reduction in cell proliferation is coupled with a
decrease in the mRNA levels of Bcl-XL and survivin (BIRC5) and an increase in the mRNA levels of Bax.

Bcl-xL is a mitochondrial membrane protein that regulates the stability of the mitochondrial internal environment (Wenshen et al. 2021). Bcl-xL is frequently overexpressed in lung cancer, hepatocellular carcinoma, and breast cancer (Wenshen et al. 2021). In breast cancer, Bcl-xL expression is linked to increased metastasis and the invasiveness of the tumor (Alam and Mishra 2021). We found that *S. Officinalis* significantly reduced Bcl-XL transcription as it was undetectable in MCF7-treated cells. Other reports showed similar findings that *Salvia aurea, S. judaica,* and *S. viscosa* significantly reduced the levels of bcl-2, and bcl-xL in human prostate cancer (DU-145) cells (Ryu et al. 2012). *Salvia Miltorrhiza* also inhibited Bcl-xL and survivin expression in MCF-7 and MDA-MB-231 human breast cancer cell lines (Sha et al. 2018). Luteolin also suppressed the expression of JAK2/STAT3 targets such as survivin, Mcl-1, and Bcl-xL in human gastric cancer cells (Song et al. 2017).

In addition to *S. Officinalis,* we found cisplatin dramatically reduced the expression of Bcl-xL to the same extent as *S. Officinalis.* This is consistent with previous findings. Cisplatin significantly reduced the expression of c-Myc and Bcl-2 but increased Bax levels in lung adenocarcinoma cell lines (Xie et al. 2019). Bax genes interact with members of the Bcl-2 protein family to activate apoptosis in response to diverse stimuli. Bax activation represents an irreversible point in the process of cell death (Lan et al. 2020).

The current results add to previous reports that show that *S. Officinalis, S. clandestina,* and *Salvia Miltorrhiza* increased the transcription level of Bax in MCF-7, and MDA-MB-231 breast cells (Nicolin, Fancellu, & Valentini 2014) breast cancer cells, mammary gland carcinoma (4T1) cells (Moghadam, Masoudi, and Monsefi 2018), and in human osteosarcoma cell line MG–63 (Z. Huang et al. 2021). Regarding cisplatin, we found that cisplatin significantly increased BAX. A similar study demonstrated that cisplatin increased Bax levels in a dose-dependent manner compared with the control in lung adenocarcinoma cell lines (Xie et al. 2019).
Survivin or baculoviral inhibitor of apoptosis repeat containing 5 (BIRC5) (Song et al. 2017) is essential for apoptosis, cell division, and cell migration/metastasis (Duffy et al. 2007). It is generally associated with a poor prognosis and a low patient survival rate (Duffy et al. 2007). The BIRC5 gene encodes the multifunctional protein survivin, which helps to build the chromosomal passenger complex in the nucleus and regulates cell division, while its presence in the cytoplasm and mitochondria helps to prevent apoptosis. In this study, we found that S. Officinalis reduced the expression of BIRC5/survivin. Other studies, however, found that luteolin and Salvia Miltorrhiza inhibited the expression of survivin in human gastric cancer cells (Song et al. 2017) and in MCF-7 and MDA-MB-231 human breast cancer cell lines (Sha et al. 2018). This difference could be due to the difference in the extract used. On the other hand, we found that cisplatin dramatically decreased the expression level of BIRC5 more than S. Officinalis. Similarly, a study showed that cisplatin (33.3 μmol/L) reduced expression of the BIRC5 gene, suggesting induction of apoptosis in the human non-small cell lung carcinoma (NCI-H460) cell line (Ballestreri et al. 2018).

Overall, we demonstrated that S. Officinalis effectively inhibited the cell growth of the MCF-7 cell line. It also induced apoptosis by reducing Bcl-xL and survivin (BIRC5) mRNA expression in MCF-7 cell line. Additionally, it increased Bax expression, indicating the involvement of the mitochondrial pathway. Taken together, these results confirm the antitumor effect of Salvia Officinalis in breast cancer in vitro.

Effects of Salvia officinalis on the expression levels of miRNAs.

MicroRNAs (miRNAs) are important players in a variety of biological processes, including cancer cell proliferation, differentiation, and migration (Tutar, Tutar, and Tutar 2014)(Vimalraj et al. 2013). They can be used as therapeutic targets in cancer intervention and treatment. JAK2 had binding sites for miR-204-5p, miR-216a-5p, and miR-101-3p/5p, according to our findings. This implies that the three miRs could directly target JAK2’s 3'UTR sequences.
MiR-101-3p showed high specificity (74.2%), sensitivity (83.8%), accuracy (79%), and area under the curve (0.814) in triple-negative breast cancer (TNBC) patient’s blood, implying that miR-101 can be used as diagnostic, prognostic, and therapeutic biomarkers in breast cancer (Mohammadi et al. 2016). Furthermore, miR-216a acts as a possible therapeutic and diagnostic target in cancer patients (Hamidi et al. 2023). Additionally, miR-204-5p is a potential prognostic biomarker of breast ductal and lobular cancers that can predict overall survival (L. Wang et al. 2014).

The genes that encode miR-101 can be found at 1p31.3 and 9p24.1. Loss of 1p31.3 area is common in a variety of cancers, including breast, stomach, and prostate cancers (Toda et al. 2020). Genetic deletions of one or both miR-101 loci are associated with glioblastoma multiforme, lung adenocarcinoma, and acute lymphocytic leukemia. Downregulation of miR-101 has a critical role in cancer cell proliferation, apoptosis, migration, invasion, blood vessel formation, and therapeutic resistance (Toda et al. 2020). Elevated miR-101 levels in ovarian cancer patients have been found to predict overall survival (Roscigno et al. 2020). Other studies showed that miR101-5p (the passenger strand of the miR101 duplex) acts as a tumor suppressor in MDAMB231 and MCF7 breast cancer cells. Several oncogenic targets regulated by miR101-5p, such as SH3 domain binding kinase 1, have been linked to breast cancer pathogenesis and oncogenesis (Toda et al. 2020). The expression of these target genes has been linked to cell proliferation, apoptosis inhibition, migration, and invasion.

Both miR-101 3p & 5p, inhibited cell proliferation and promoted apoptosis (L. Wang et al. 2014). We here focused on miR-101-5p; its expression was significantly lower in MCF-7 cell lines compared to HSF cells. This is in line with previous research findings, which showed that miR-101 expression was reduced in seven breast cancer cell lines (BT-483, T47D, MCF-7, SKBR3, BT-474, MDA-MB-435, MDA-MB-231, and MDA-MB-468). miR-101 expression was also low in triple-negative breast cancer samples and HER2-positive breast cancer (Xiaoyun Liu et al. 2022). This demonstrates that miR-101-5p has tumor-suppressive properties (Normann et al. 2022; Xiaoping et al. 2013). Both *S. Officinalis* and cisplatin significantly reduced the expression of miR-101-5p in our study.
MiR-101 deficiency promotes cancer cell proliferation by increasing the expression of several oncogenes. According to Zhu et al. (Zhu and Li 2017), overexpression of miR-101 increases induced mitochondrial apoptosis in papillary thyroid cancer by targeting c-met and MCL-1, indicating that miR-101 has the potential for cancer treatment.

In the current research, we also reveal the downregulation of miR-204 in MCF-7 cells compared to normal cell lines. In addition, S. Officinalis increased the expression of miR-204-5p, while cisplatin had no effect.

In normal tissues, miR-204-5p plays a significant role in eye development, lipogenesis, and osteogenesis (Yang et al. 2023). It is also a key tumor suppressor as it regulates cancer cell proliferation, metastasis, apoptosis, and autophagy. MiR-204-5p plays a tumor suppressor role, and its decreased expression promotes tumorigenesis and progression (Yang et al. 2023). It is also decreased in breast cancer (W. Li et al. 2014; Liang et al. 2019; Roukens et al. 2021).

MiR-204-5p levels are associated with improved survival in breast cancer patients, and its expression is significantly downregulated in breast cancer tissues (Hong et al. 2019)(W. Li et al. 2014). Increasing miR-204-5p resulted in Inhibiting cell viability, proliferation, and migration in human breast cancer (MDA-MB-231) and (4T1) murine breast cancer. Other reports, however, point to its pro-proliferative impact in breast cancer cells and prostate cancer cells, indicating its dual regulatory role (H. Lee et al. 2016; T. Li, Pan, and Li 2016).

MiR-216a-5p is downregulated in pancreatic, oral squamous cell carcinoma, colorectal, and small-cell lung cancers (Sun et al. 2018)(Roscigno et al. 2020; Chai and Yang 2019; Hou et al. 2012). Serum miR-216a levels are significantly low in breast cancer patients (Xie et al. 2019). This was consistent with our results that showed the downregulation of miR-216-5p in MCF-7 cell lines compared to normal cell lines.
We demonstrated that miRNA expression differs in normal and breast cancer cells, as well as after treatment. Other research has linked the differential expression of miRNAs to molecular subtypes, genetic mutations, and cell types. A study, for example, found that cell type specificity may influence miRNA expression. (Lieber 2013)

In this study, we show that S. Officinalis reduced the expression of miR-216a-5p compared to cisplatin on MCF-7 cell lines. These findings contradict previous findings that S. miltiorrhiza increased miR-216b expression in both U266 and U937 cells (C. Kim et al. 2018). This could be due to the differences in cell lines used.

**Overexpression of miR-216a-5p suppresses JAK2, BAX, BIRC5 & Bcl-xL expression**

Increasing the level of expression of miR-216-5p reduced cell proliferation in tumor tissues (S. Wang, Chen, and Tang 2014) and contributes to breast cancer development by releasing pro-inflammatory molecules such as interleukin 6 (IL-6) (Zhang et al. 2019). However, this contradicted our results. We found that miR-216a-5p mimics had no effect on cell viability on MCF-7 cell lines after 24 and 48 hrs. Similarly, Other studies showed that miR-216a-5p overexpression promoted cell proliferation in Diabetic nephropathy (C. Huang et al. 2019).

The JAK2 baseline expression in MCF7 was significantly upregulated compared to that of the HEK cell line, which indicates that it is overexpressed in cancerous cells. Previous studies showed that JAK2 expression was increased in cervical cancer tissues and breast cancer tissues and breast cell lines (MCF-7 and MDA-MB-231) (L. Wang et al. 2014). In this study, we found that miR-216a-5p mimics reduced JAK2 mRNA expression by 80%. These findings were consistent with previous research. A previous study found that miR-216a significantly decreased JAK2 mRNA levels while anti-miR-216a significantly increased JAK2 mRNA levels, indicating that miR-216a directly targeted JAK2 in pancreatic cancer cells (L. Wang et al. 2014). In our work, we found that S. Officinalis significantly reduced the expression level of JAK2 mRNA compared to that following cisplatin treatment and the levels after miR-216a-5p overexpression. These results show that miR216 may possibly be used as a breast cancer treatment tool.
Furthermore, the overexpression of miR-216a increased apoptosis, caspase-3/8 activity, Bax expression, and p53 protein expression in MCF-7 cells (Zhang et al. 2019). These findings are in line with the current results that the overexpression of miR-216a-5p in MCF7 increased the expression level of BAX and reduced the expression level of BIRC5 and Bcl-xL. It also significantly reduced the expression of JAK2 mRNA.

Figure 4.1 The effect of cisplatin, *S. officinalis* and miR-216a-5p mimics on the expression level of Bcl-xL. BAX & BIRC5.

Targeting JAK2/STAT3 pathway. cisplatin, *S. officinalis*, and miR-216a-5p mimic and reduce the expression of Bcl-xL and Survivin (BIRC5). However, they increase the expression level of Bax, suggesting the involvement of *S. Officinalis* in the mitochondrial (intrinsic) pathway.
Table 6. A summary of the effects of *S. Officinalis*, cisplatin, and miR-216a-5p mimics.

<table>
<thead>
<tr>
<th>Gene</th>
<th><em>Salvia Officinalis</em></th>
<th>Cisplatin</th>
<th>miR-216a-5p mimics</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2</td>
<td>(90%)</td>
<td>(30%)</td>
<td>(80%)</td>
</tr>
<tr>
<td>BAX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIRC5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-xL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced Cell proliferation</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
Conclusion and future perspectives

To summarize, dried leaf of *S. Officinalis*, with its rich content of Linolein and Apigenin, inhibited MCF-7 cell proliferation with an IC50 range of 5.123 to 6.345 mg/mL when compared to cisplatin (IC50=20 ug/ul). *S. Officinalis* was also found to be safe for human skin fibroblasts, implying that it has anticancer activity while being less harmful to normal cells.

*S. Officinalis* significantly reduced JAK2, Bcl-xL, and BIRC5 mRNA expression, while increasing BAX expression. JAK2's 3' UTR contains theoretical binding sites for miR-101-5p, miR-216a-5p, and miR-204-5p. The expression of miR-101-5p and miR-216a-5p were reduced by *S. Officinalis*, while the plant extract had no significant effect on miR-204-5p. Overexpression of miR-216a-5p had no effect on the cell viability of MCF-7 cells, dramatically decreased the expression of JAK2, BIRC5, and Bcl-xL, and increased the expression of BAX.

We confirm that *S. Officinalis* exerts its anticancer activity via JAK/STAT3 pathway. More studies are needed to investigate the role of other microRNAs and test the efficacy of *S. Officinalis* in reducing tumor metastasis *in vivo*. 
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The data show the genotype-tissue expression (GTEx) of JAK2 Homo sapiens Janus kinase 2 (JAK2), mRNA, Ensembl gene ID: ENSG00000096968.13, GENCODE biotype: protein_coding. The median levels of gene expression in 52 tissues and 2 cell lines are shown in this track, which is based on RNA-seq data from the GTEx final data release. (TPM stands for transcripts per millions)

For investigations on the connection between genetic variation and gene expression in various human tissues, GTEx was established as a sample and data repository. Using the National Cancer Institute Biorepositories and Biospecimen, tissue samples were collected in accordance with the GTEx standard operating procedures for informed consent and tissue collection. To characterise and confirm the organ source, pathologists examined all tissue samples. Tissue samples were stabilised without having their biomolecules cross-linked using the Qiagen PAXgene non-formalin tissue preservation kit.
The GTEx Laboratory, Data Analysis and Coordinating Centre (LDACC) at the Broad Institute carried out the RNA-seq. An unstranded polyA+ library was made using the Illumina TruSeq procedure and sequenced on the Illumina HiSeq 2000 and HiSeq 2500 platforms to produce 76-bp paired end reads with a goal of 50M (the actual coverage attained was around 82M total reads).

By merging overlapping exon intervals and reading through transcripts, excluding retained intron, and then excluding exon intervals overlapping between genes, a bespoke isoform collapsing approach was used to create gene annotations. After filtering for distinct mapping, appropriate pairing, and exon overlap, the RNA-SeQC programme (v1.1.9) was used to determine the TPM (transcripts per millions) levels of gene expression.

We found that the Artery - Tibial has the highest median expression, 68.89 TPM; however, the breast mammary tissues' median expression was 459 TPM.
The figure shows the predicted miRNAs target sites from Target scan 7.2

The data show the chromosome location: 4985245 - 5128183 and main target sites of nine miRNA in 3’ UTR JAK2: ENST00000381652.
Figure shows the main target site of the human JAK2 ENST00000381652.3 3' UTR length: 3158. (obtained from Target scan 8.0)

We also found the 3'-UTR profiles, which indicate the location and usage of mRNA cleavage and polyadenylation sites (Human JAK2 ENST00000381652.3). 3P-seq tags from multiple cell lines or tissues were normalized to each other (to account for variable sequencing depth), and then aggregated into one consensus set of counts. Normalized 3P-seq tags were assigned to the representative 3' UTR of each stop codon and summed (as indicated at the left side of the profile) to provide quantification for the usage of that stop codon. The sites of three miRNAs showed a high probability of preferential conservation.
The table illustrates Human JAK2 (ENST00000381652.3) 3’ UTR miRNA broadly conserved among vertebrates

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Conserved sites</th>
<th>Cumulative weighted context++ score</th>
<th>Total context++ score</th>
<th>Aggregate PCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-101-3p.1</td>
<td>(8-mers)</td>
<td>-0.33</td>
<td>-0.34</td>
<td>0.76</td>
</tr>
<tr>
<td>miR-216a-5p</td>
<td>(8-mers)</td>
<td>-0.19</td>
<td>-0.19</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>miR-204-5p/211-5p</td>
<td>(8-mers)</td>
<td>-0.32</td>
<td>-0.35</td>
<td>0.46</td>
</tr>
<tr>
<td>miR-101-3p.2</td>
<td>(8-mers)</td>
<td>-0.11</td>
<td>-0.13</td>
<td>0.37</td>
</tr>
<tr>
<td>miR-144-3p</td>
<td>(8-mers)</td>
<td>-0.21</td>
<td>-0.22</td>
<td>0.37</td>
</tr>
<tr>
<td>miR-375</td>
<td>(8-mers)</td>
<td>-0.18</td>
<td>-0.18</td>
<td>0.58</td>
</tr>
<tr>
<td>miR-135-5p</td>
<td>(8-mers)</td>
<td>-0.32</td>
<td>-0.32</td>
<td>0.43</td>
</tr>
</tbody>
</table>

The table displays miRNA families and is sorted by cumulative weighted context++ score. The context++ score indicates the likelihood of successfully targeting a specific target. The weighted context++ score (WCS) takes into account the abundance of each 3’-UTR tandem isoform in which the site exists, as determined by the UTR profile (a collection of 3P-seq datasets from the same species). The WCS for a specific site is the sum of 14 feature contributions. 8-mers, which represent conserved sites, demonstrated a high likelihood of preferential conservation.

Furthermore, according to target scan 8.0, PCT (the probability of conserved targeting) has been calculated for all highly conserved miRNA families. By decreasing aggregate PCT, predicted targets of a miRNA family can be sorted, as described in Friedman et al., 2009.

We chose three miRNAs (miR-101-3p.2, miR-215a-5p, and miR-204-5p) to proceed with the experiments based on previous findings and other studies.