School of Sciences and Engineering

The Effect of EPHEDRA FOEMINEA on Human Bone Osteosarcoma U2OS Cell

Viability and Migration

A Thesis Submitted to

The Master of Biotechnology Program

In Partial Fulfillment of the Requirements for

The Degree of Master of Science in Biotechnology

By: Mpingirika Eric Zadok

Under Supervision of

Dr. Asma Amleh

Associate Professor, Biology Department,

The American University in Cairo

December/2018
The American University in Cairo

School of Sciences and Engineering (SSE)

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Thesis Committee Supervisor/Chair ______________________________________________________

Affiliation __________________________________________

Thesis Committee Reader/Examiner ___________________________________________________

Affiliation __________________________________________

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Affiliation __________________________________________

Thesis Committee Reader/External Examiner ____________________________________________

Affiliation __________________________________________

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DEDICATION

To my dear late mother, Sara Nalani who lost the battle to cancer in 2004; thank you for nurturing me into the person I’ve become today.
Acknowledgements

I would like to thank Dr. Asma Amleh for her mentorship and guidance throughout my graduate studies. I am grateful for her selfless dedication to making this project a success. I thank all professors in the Biotechnology Master program at AUC for their relentless endeavor in making me a better scientist and researcher; most notably I acknowledge Dr. Walid Fouad for teaching how to think critically.

I am grateful to both Dr. Rami Arafeh and Mr. Ahmed El Hosseiny who were instrumental in respectively contributing towards the processing of *E. foeminea* crude extracts used in this study and in silico analysis of U2OS gene expression data.

Many thanks go out to Dr. Asma Amleh research team (Myret Ghabriel, Mennatallah Elfar, Sheri Saleeb, Nancy Hassanein, Noha Saad, Mennatallah Ghoraba, Khaled Abdel Raouf, Rowan Bahaa, Omnia Abdelraheem, Alaa Farag and Naela Adel Saleh) for sharing their knowledge and support all through the course of this project.

My appreciation goes out to my dear friends (Logayn Tarek, Ogwang Joel and Mugwanya Muziri) for their tremendous support throughout the ups and downs of grad school.

Finally, I thank The American University in Cairo (AUC) for providing the African Graduate Fellowship which has facilitated my study at AUC in addition to providing the research grant without which this study would have been impossible.
Abstract

Although advancement has been made in the development of cancer treatments, contemporary treatments still present significant challenges such as low effectiveness and adverse side effects. There is thus a critical need to continuously develop new and more effective drugs against cancer. Herbal plants serve as a potential source for a wide variety of complex compounds with probable anticancer activity. *E. foeminea* is an herb whose use in the Middle East recently gained popularity as a remedy for cancer. There is however minimal empirical evidence regarding the anticancer effects of *E. foeminea*. In this study, the effect of *E.foeminea* ethyl acetate, ethanol and water extracts on morphology, viability, migratory ability and gene expression of U2OS osteosarcoma cells was examined. U2OS viability, migratory ability and the steady-state mRNA levels of genes involved in these processes were respectively studied using MTT assay, wound healing assay and reverse transcriptase PCR (RT-PCR). Results showed that all tested extracts significantly reduced U2OS percentage viability in a manner dependent on both dose and time with varying potencies; the least half maximal inhibitory concentration (IC50) recorded was that of the water extract after 48h incubation (30.761±1.4 µg/ml) followed by the ethyl acetate extract after 72h incubation (80.35±1.233 µg/ml) and finally the ethanol extract after 48h incubation (97.499±1.188 µg/ml). Ethanol extract significantly reduced U2OS percentage wound closure while both ethanol and water extracts significantly reduced the steady-state mRNA expression of Beta-catenin and its downstream targets, Twist1 and RUNX2, which are critical in promoting both proliferation and cell migration in osteosarcoma. These results suggest that *E. foeminea* decreases U2OS cell viability and migration by modulating the expression of key genes involved in regulating these processes.
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1. **ANOVA**: Analysis of variance
2. **Ap-1**: Activator protein 1
3. **BAX**: BCL2 Associated X
4. **B-catenin**: Beta catenin
5. **BCL-2**: B-cell lymphoma 2
6. **Cdna**: Complementary Deoxyribonucleic acid
7. **C-MYC**: Avian myelocytomatosis viral oncogene homolog
8. **Cox-2**: Cyclooxygenase-2
9. **DMEM**: Dulbecco's Modified Eagle's medium
10. **DMSO**: Dimethyl sulfoxide
11. **DNA**: Deoxyribonucleic acid
12. **E.foeminea**: *Ephedra foeminea*
13. **EDTA**: Ethylenediaminetetraacetic
14. **EtOAc**: Ethyl Acetate
15. **EtOH**: Ethanol
16. **G2 phase**: Gap 2 phase
17. **GAPDH**: Glyceraldehyde 3-Phosphate Dehydrogenase
18. **GEO**: Gene Expression Omnibus
19. **H2O**: Water
20. **HCT116**: human colorectal carcinoma cell line
21. **HL60**: Human promyelocytic leukemia cell line

22. **HOS**: Human Osteosarcoma cells

23. **IC50**: Half maximal inhibitory concentration

24. **LRP**: Low density lipoprotein receptor-related protein

25. **M phase**: Mitotic Phase

26. **MAPK**: Mitogen-activated protein kinases

27. **MMP 9**: Matrix metalloproteinase 9

28. **MMP1**: Matrix metalloproteinase 1

29. **MMP2**: Matrix metalloproteinase 2

30. **mRNA**: Messenger ribonucleic acid

31. **MTT**: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

32. **N-cadherin**: Neural cadherin

33. **NF-KB**: Nuclear factor kappa-light-chain-enhancer of activated B cells

34. **p53**: Tumor protein p53

35. **PBS**: Phosphate-buffered saline

36. **PCR**: Polymerase Chain Reaction

37. **RNA**: Ribonucleic acid

38. **RT-PCR**: Reverse transcription-polymerase chain reaction

39. **RUNX-2**: Runt-related transcription factor 2

40. **SAOS**: Sarcoma osteogenic

41. **TWIST-1**: Twist Family BHLH Transcription Factor 1

42. **Wnt/B-catenin**: Wingless/Integrated/beta catenin
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Chapter 1. Literature Review

1.1 Introduction

Accounting for approximately a sixth of all deaths worldwide, the World Health Organization ranks cancer as the second leading cause of death in the world. With 14 million new cases recorded in 2012, a 70% increase is such incidences is expected within the next twenty years (WHO, 2017). Although progress has been made in cancer treatment, contemporary cancer remedies including surgery, chemo- and radiotherapy have been shown to be both expensive and ineffective. In addition, a number of adverse side effects in cancer patients receiving either chemo- or radiotherapy have been observed. Furthermore, some cancers have a tendency to develop resistance to drugs used in chemotherapy (Dai et al., 2016). Cancer research should thus make it an aim to continuously develop more affordable and effective treatments (Dai et al., 2016). Herbal plants are a potential source of diverse and complex compounds such flavonoids, phenols, alkaloids, lectins and terpenes that could possess probable anticancer properties. Several plant derived anticancer agents like vinca alkaloids have been approved by the United States Food and Drug Administration (FDA) while others agents like flavopiridol, are under clinical development (Cragg & Newman, 2005).

_Ephedra foeminea_ (E. foeminea) is a shrub belonging to the _Ephedraceae_ family (IUCN, 2010). Plants belonging to the ephedra genus have previously been used as a source of ephedra alkaloids, which makeup the active components of the drug ephedrine. Ephedrine has been used both as a stimulant and a weight loss agent, dietary supplements containing herbal _Ephedra_ have however been reported to be associated with psychosis, severe depression, agitation, hallucinations, sleep disturbance, suicidal ideation, and several addiction symptoms (Maglione et al., 2005). Ephedra has also been shown to cause strokes and heart problems, prompting a ban in the United States of
drugs containing Ephedra, in 2004. Ephedra alkaloids were however not detected in *E. foeminea* (Ibragic & Sofic, 2015).

Although very little is known about the use of *E. foeminea* to treat cancer, its use among Middle Eastern patients has recently become popular due to the belief that the herb possesses curative properties for cancer (Ben-Arye et al., 2016).

A previous study (Ben-Arye et al., 2016) showed that the boiled water extract of *E. foeminea* showed no significant effect on MDA-MB231 & SKBR3 breast cancer cell lines. However, another study (Maayan et al., 2017) showed that *E. foeminea* ethanol, water and fruit juice extracts reduced the viability of these cell lines. Further still, the study showed that *E. foeminea* extracts also reduced the viability of A549 lung carcinoma and HCT116 colorectal carcinoma cells (Maayan et al., 2017). This discrepancy thus warrants the further study of *E. foeminea*’s effect on different types of cancer cells along with the utilization of varying extraction methods. It has been shown that different extraction methods variably affect extract potency (Azwanida, 2015). Also, different cell lines are known to have differential responses to a similar extract or testing agent (Wilding & Bodmer, 2014) . This study therefore utilized the maceration technique to produce *E. foeminea* crude extracts using ethyl acetate, ethanol and water as solvents. The effect of each extract on the viability of U2OS cells was determined using MTT assay while the wound healing assay was employed to study the extracts’ effect on U2OS cell migration ability. Differential gene expression of several markers such as *Beta-catenin* (*B-catenin*), *Twist-1* and *RUNX2* that regulate key pathways involved in both cell proliferation and migration was tested. All extracts tested in this study significantly reduced U2OS cell viability in a manner dependent on both dose and time. In addition, all extracts were able to reduce U2OS migration ability however only the ethanol extract showed statistically significant results. Furthermore, all extracts downregulated the steady-
state mRNA expression of several genes involved in cell proliferation, apoptosis and migration with statistically significant results observed for both ethanol and water extracts. These results suggest that *E. foeminea* most probably affects U2OS proliferation and migratory ability by modulating key genes involved in the regulation of these processes.

### 1.2 Cancer Treatment

Currently, the most popular methods for cancer treatment include surgery, chemotherapy and radiotherapy (Dai et al., 2016). Briefly, surgery is used to gain access to tumors by cutting through skin, muscle or bones in order to either completely excise or de-bulk the tumor. Chemotherapy on the other hand involves the use of drugs to kill or slow the growth of cancer cells while radiotherapy utilizes high radiation doses to do the same by damaging cancer cells’ DNA (National Cancer Institute, 2017). In addition to being both ineffective and expensive, these treatments have been shown to have numerous setbacks; many times, surgery ceases to be an option due to late diagnosis. Furthermore, both chemo- and radiotherapy possess serious side effects and complications including: fatigue, diarrhea, nausea, vomiting, hair loss, pain and respiratory and liver complications among others. Also, cancer cells have tend to develop gradual resistance towards these treatments (Qi et al., 2010).

There is therefore a need to continuously develop new, effective and more affordable anticancer drugs. Herbal plants present a suitable alternative for cancer treatment. As of 2014, 90 out of 121 drugs approved for cancer treatment were associated with herbal plants; to add, studies show that between 1981 and 2002, 48 out of 65 new registered cancer drugs were obtained from natural products; and 25% of these were associated with medicinal plants. Herbal medicine for cancer treatment is mainly used a preventive measure against both primary occurrence and recurrence of
cancer in addition to boosting the body’s immunity and alleviating side effects resulting from contemporary cancer treatment (Safarzadeh, Sandoghchian Shotorbani, & Baradaran, 2014).

1.3 Herbal Plants in Cancer Treatment

A number of herbal derived compounds that have been shown to possess anticancer activity. These include:

**Phenolic compounds;** phenols are a class of compounds that contain a hydroxyl group bonded directly to an aromatic ring (Balasundram, Sundram, & Samman, 2006). Studies have shown that various herbal polyphenol complexes, common in food are cytotoxic to several tumors; their cytotoxic mechanism is mainly via apoptosis induction. In addition to increasing chemical sensitivity to doxorubicin, research shows that diferolyl methane, a phenol compound isolated from curcuma species rhizome possesses anticancer activity against colorectal, pancreas, gastric and prostate cancers in pre-clinical trials (Taraphdar, Roy, & Bhattacharya, 2001). Furthermore, both phenol complexes from ginger rhizome (Taraphdar et al., 2001) and resveratrol from grapes have been shown to induce apoptosis in number of cancer cell lines. Resveratrol for example showed cytotoxicity against HL60 and T47D breast carcinoma cells (Wheat & Currie, 2008).

**Flavonoids;** flavonoids are a group of polyphenolic compounds with a basic diphenylpropane structure (C₆C₃C₆) (Rice-Evans, Miller, & Paganga, 1996). Flavonoids like genistein have shown apoptosis induction in human pro-mayelotic HL-60 leukamic cells (Qi et al., 2010). Genistein was further shown to inhibit angiogenesis in addition to triggering cell cycle arrest at the G2/M phase (Wheat & Currie, 2008). On the other hand biocalein, a flavonoid in sho-saiko-to herbal medicine
causes apoptosis in human hepato-cellular carcinoma (HCC) cell lines. Further still, quercetin was also shown to induce apoptosis via the caspase-9 pathway (Taraphdar et al., 2001).

**Alkaloids;** alkaloids are cyclic naturally occurring compounds that possess nitrogen in its negative oxidation state (Wink, 1998). Vinca alkaloids, derived from *Catharanthus roseus* were used to develop vinblastine and vincristine chemotherapeutic drugs that are currently in clinical use to treat spleen and liver cancer in addition to childhood leukemia. More to this, the alkaloid, camptothecin isolated from the Chinese tree, *Camptotheca acuminata*; was shown to be a potent anticancer agent that inhibits type 1 DNA topoisomerase enzymes thus interfering with cell cycle progression. Since its discovery, several camptothecin derivatives such as topotecan and irinotecan were clinically approved for use against several cancer types (Avendaño & Menéndez, 2015).

**Lectins;** lectins refer to a group of proteins that are able to bind to carbohydrates and are of both plant and animal origin (Goldstein & Hayes, 1978). The plant *Viscum album* L which has for long been used in adjuvant cancer therapy has been shown to contain mistletoe related lectins that stimulate the immune system by increasing numbers of both neutrophils and natural killer cells. Also, KML-C, another mistletoe related lectin, isolated from *Viscum album* C. (Korean mistletoe) was shown to possess both cytotoxic and apoptotic effects against cancer cells in vitro mediated by nucleases dependent on both calcium and magnesium ions (Taraphdar et al., 2001).

**Terpenoids;** terpenoids are the largest and most structurally diverse group of naturally occurring compounds that are common in plants. They result from the condensation of carbon isoprene units and lack both sulfur and nitrogen (Wink, 2010). Studies have shown that the sesqui-terpenoid, xanthorrhizol, isolated from *Curcuma xanthorrhiza* Roxb inhibits tumor formation and development in several in vivo studies by suppressing NF-KB anti-apoptotic signaling; moreover,
using a mouse lung metastasis model, xanthorrhizol was shown to exert anti-metastatic activity by downregulating both cyclooxygenase-2 (cox-2) and matrixmetallopeptidase-9 (MMP-9) (Cheah et al., 2008). In addition, the diterpenoid, taxol, isolated from the bark tissue of *Taxus brevifolia* (Pacific yew) is a clinically proven chemotherapeutic agent with outstanding potency against a various cancers such as ovarian and breast cancers (Wink, 2010).

1.4 Mechanisms of Anticancer Herbal Medicine

Broadly, anticancer herbal medicines are classified into two classes depending on their mode of action. These include immunomodulation and chemo-preventive herbs. Immunomodulation herbs are those herbs that boost immune system activity, this is of key importance since cancer patients tend to have diminished innate immunity in addition to tumor cells’ ability to evade the immune system. Chemo-preventive herbal medicines on the other hand utilize natural chemical complexes to either interfere with or prevent tumor development mainly through apoptosis induction (Safarzadeh, Sandoghchian Shotorbani, & Baradaran, 2014).

More specifically, anticancer herbal medicines utilize a number of mechanisms to produce their cytotoxic effects against tumor cells. These include modulation of cell signal transaction pathways such as Ap-1, NF-KB, Cox-2 and MAPKs that play roles in angiogenesis and proliferation of cancer cells. Another mechanism is by cell cycle modulation whereby checkpoints in tumor cells are reactivated thus leading to a halt in their proliferation. In addition, herbal anticancer medicines such as Vinca alkaloids can interfere with microtubule formation leading to a halt of mitosis at metaphase hence resulting in apoptosis. Finally, certain herbal anticancer drugs inhibit topoisomerases which are involved in DNA replication. Captothecins and epodo phylotoxins have shown inhibition for topoisomerase 1 and 2 respectively (Safarzadeh et al., 2014).
Currently, more than 60 herbal chemical complexes are under research for their anticancer properties; also, since almost half of all existing medicines are plant derived, it is evident that plants, are worthy of further investigation as sources of antitumor compounds (Safarzadeh et al., 2014). The challenge of drug resistance by cancer cells can be alleviated by the development of new and functionally effective drugs. The enormous degree of both complexity and diversity of plant derived compounds can be utilized to develop novel anticancer drugs (Cragg & Newman, 2005). Plant derived anticancer drugs can also be used alongside conventional cancer treatments to ameliorate the negative side effects of popular treatments (Qi et al., 2010).

1.5 U2OS Cell Line

The U2OS osteosarcoma cell line was utilized in this study. The cells were originally isolated from the tibia of a 15-year old Caucasian female. The cells are moderately differentiated and possess an epithelial morphology; chromosomes are highly altered and exist in the hyper-triploid range (ATCC, 2016).

1.6 Osteosarcoma

Osteosarcoma is the most common primary malignant bone tumor prevalent in both children and adolescents. It accounts for approximately 5% of all pediatric malignancies and has a global incidence of about 4.5 cases per 1 million individuals less than 24 years per year. Osteosarcoma treatment is mainly a combination of both surgery and chemotherapy which have improved the survival rate of patients by about 70%. Common chemotherapy drugs used for osteosarcoma treatment include doxorubicin, cisplatin and methotrexate (American Cancer Society, 2018).
1.7 Key Markers Involved in Osteosarcoma

**Wnt/B-catenin signal transduction pathway:** In this study, the expression of a key regulator of proliferation in osteogenic cells, beta-catenin, was assessed. Beta-catenin steady-state mRNA was showed to be down regulated by *E. foeminea*. Beta-catenin is regulated by the Wnt signaling cascade in which Wnt glycoproteins bind both Frizzled receptors and LRP 5/6 co-receptors initiating a cascade in which through disheveled ultimately leads to phosphorylation of the beta-catenin destruction complex. Phosphorylation marks b-catenin for degradation and vice versa; un-phosphorylated b-catenin migrates to the nucleus and acts as coactivator, promoting the transcription of several downstream oncogenes such c-myc and cyclin-D1. Accordingly, aberrant, Wnt signaling contributes to the development of several forms of cancer including osteosarcoma; studies have further shown that beta-catenin is relatively highly expressed in osteosarcoma (Kleinerman, 2014).

**RUNX2 pathway:** RUNX2 is a transcription factor that has been shown to be a major regulator of both osteogenic differentiation and proliferation. As a controller of both differentiation and growth, RUX2 expression has been shown to inhibit the proliferation of both normal osteoprogenitor and pre-osteoblast cells at the late G1 phase of the cell cycle. On the other hand however reports show that RUNX2 equally has the ability to promote cell proliferation at the early G1 phase (Lucero et al., 2013). RUNX2’s promotion or inhibition of cell proliferation has thus been shown to be specific to cell type; whereas RUNX2 mainly inhibits growth in both osteoprogenitor and pre-osteoblast cells, it has been shown to promote cell proliferation in chondrocytes of RUNX2 null mice (Jitesh Pratap et al., 2003). Further still, aortic endothelial cells showed increased proliferation when RUNX2 was ectopically expressed (Sun, Vitolo, Qiao,
Anglin, & Passaniti, 2004). Such dual functionality of RUNX2 thus suggests that RUNX2 protein could act as either a tumor suppressor or an oncoprotein. Indeed, osteosarcoma biopsies having high RUNX2 expression have been shown to have adverse tumor characteristics such as greater tumorigenicity, tumor progression and metastases and has further been shown to control several cancer related genes. A previous study has shown that RUNX2 retains its anti-proliferative ability in osteosarcoma cell lines including U2OS; in these cells therefore, it is proposed that although RUNX2 is relatively highly expressed, it is maintained at levels that avoid tumor suppression (Lucero et al., 2013).

**p53 apoptotic pathway:** Key to the success of cancer drug therapies is their ability to induce programmed cell death including apoptosis in cancer cells. The effect of *E.foeminea* extracts on genes involved in apoptosis was thus studied. p53 and Bax are two genes the play key roles in the regulation of apoptosis. Most drugs that induce apoptosis do so via the intrinsic apoptotic pathway in which apoptosis is initiated by intracellular signals that ultimately lead to the opening of the mitochondrial inner membrane, loss of potential at the mitochondrial transmembrane and finally release of pro-apoptotic proteins into the cytoplasm. These mitochondrial changes are regulated by the Bcl-2 protein family which is composed of both pro- and anti-apoptotic members (Elmore, 2007). BAX belongs the pro-apoptotic group of the Bcl-2 family. Reports show that Puma, a Bcl-2 family member is induced by p53 and that it’s over expression most probably mediates BAX expression, structural change and mitochondrial translocation in addition to release of cytochrome c mitochondrial membrane potential reduction. Other reports have further shown that p53 is able to directly activate BAX and initiate apoptosis (Oren, 2003).
1.8 Hypothesis

This study hypothesizes that *E. foeminea* ethyl acetate, ethanol and water extracts significantly affect U2OS cell viability, migration and gene expression.

1.9 Aim of Study

To address this hypothesis, the main aim of this study was to assess the effect of *E. foeminea* ethyl acetate, ethanol, and water extracts on morphology, viability, migration, and gene expression of U2OS osteosarcoma cells.

This aim was achieved through four specific aims:

**Specific aim 1** involved the preparation of *E. foeminea* crude extracts using ethyl acetate, ethanol and water solvents by the method of maceration.

**Specific aim 2** involved examining the effects of prepared *E. foeminea* extracts on U2OS viability using the MTT assay.

**Specific aim 3** entailed studying the effects of *E. foeminea* extracts on the morphology and migratory ability of U2OS cells by respectively using microscopic observation and the scratch wound healing assay.

**Specific aim 4** was to study the effect of *E. foeminea* extracts on U2OS gene expression by reverse transcriptase PCR (RT-PCR).
Chapter 2. Methodology

2.1 Plant material harvest

*Ephedra foeminea* Forssk. (Ephedraceae) aerial parts (stems) were collected in July at the flowering stage from Hebron city, Lat: 31.538629, Lon: 35.085769. A voucher specimen was preserved for identification in the Biotechnology Research Center at the Palestine Polytechnic University. Identification of the plant was carried out referring to the Flora of Israel Online by Prof. Avinoam Danin. ([http://flora.org.il/en/plants/ephfoe/](http://flora.org.il/en/plants/ephfoe/)).

2.2 Crude extract preparation

Plant parts were dried at room temperature without exposure to direct sunlight. Stems were cut into small pieces (1.0 to 2.0 cm) then ground with electric grinder into fine powder. After sieving, the powder was separated from the residual fibers then one gram of the powder was mixed with 30.0 ml of the following analytical grade solvents; deionized water, absolute ethanol and ethyl acetate. After 24 hours of continuous shaking (170 rpm), the mixture was filtered and the filtrate was evaporated in the fume hood until totally dried. Extraction yield was calculated after the solvents were totally evaporated. The dried extracts were dissolved in DMSO to make stock solutions of 150 mg/ml; from these stock solutions, a series of working concentrations (1, 0.5, 0.25, 0.13, 0.06 and 0.03 mg/ml) were prepared by serial dilution using complete DMEM cell culture media.

2.3 Cell Culture

The cell line used for this study was the U2OS human bone osteosarcoma cell line (kindly provided by Dr. Andreas Kakarougkas, Department of Biology, AUC). Cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium) (Invitrogen, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (100 units/ml) and streptomycin (100 mg/ml) from...
Invitrogen. Cells were cultured at 37°C and 5% CO₂ in a humidified Hera cell CO₂ incubator from Thermo-fisher. Regular splitting of the cells was performed before they reached confluency. Splitting was performed by washing cells with phosphate buffer saline (PBS), and detaching them using 0.05% Trypsin-EDTA solution (Invitrogen, USA). Visualization of cells was done using the Olympus IX70 inverted microscope.

2.4 Trypan Blue Exclusion Assay

All cell counts were done using trypan blue exclusion assay. It involved withdrawing 10 µl from the cell suspension whose cell count was to be determined and mixing it with 10µl of 0.4% w/v trypan blue (Serva, Germany). This was followed by loading 10 µl of the mixture in each chamber of the hemocytometer (Hauser Scientific, USA). The cells in each chamber were counted and the number of either viable or non-viable cells per milliliters determined according to the equation below (Strober, 2001):

\[
\text{Viable cells/ml} = (\text{Total number of viable cells/total number of squares counted}) \times \text{dilution factor} \times 10,000
\]

\[
\text{Non-viable cells/ml} = (\text{Total number of non-viable cells/total number of squares}) \times \text{dilution factor} \times 10,000
\]
2.5 U2OS Doubling Time

U2OS doubling time was determined by culturing U2OS cells in a 6-well plate over a period 6 days. Viable cell counts were made on each consecutive day using trypan blue exclusion assay. At the end of this period, a growth curve of viable cell count against time was generated and doubling time determined using the equation below obtained from the American Type Culture Collection (ATCC) website (ATCC, 2014);

\[ DT = T \ln 2 / \ln \left( \frac{X_e}{X_b} \right) \]

Where,

- \( DT \) is the doubling time.
- \( T \) is the total time during which exponential growth occurs.
- \( X_b \) is the cell number at the beginning of exponential growth.
- \( X_e \) is the cell number at the end of the exponential growth.

2.6 MTT Cell Viability Assay

The viability of U2-OS cells incubated with *E. foeminea* extracts for 24, 48 and 72 hours was determined using the MTT viability assay. Viable cells reduce MTT reagent 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (Serva, Germany) to form a purple color by mitochondrial dehydrogenase enzymes (Riss et al., 2004). U2OS cells were incubated with *E. Foeminea* extracts in a 96-well plate for either 24, 48 or 72 hours. The culture media was aspirated and 100\( \mu \)l of fresh media containing 20\( \mu \)l of MTT solution (5mg/ml) was added per well and incubation done for 4 hours. The media was discarded after incubation and replaced with 100 \( \mu \)l of Dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) per well to solubilize the purple formazan
crystals formed as a result of MTT reduction. The color change was quantified by taking absorbance readings at 570nm using SPECTRO star nano microplate reader (BMG LABTECH). The percentage cell viability was calculated by expressing the absorbance of the treated wells as a percentage of the untreated wells.

2.7 Scratch Wound Healing Assay

The scratch wound healing assay was done to test the cell migration ability of U2-OS cells when incubated with *E. Foeminea* extracts. U2OS cells were seeded at a density of 3x10^5 cells per well in a 6-well plate and incubated till cells were about 90% confluent. At this point, two perpendicular scratches were made in the cell monolayer with the help of a sterile 20 µl pipette tip. Cells were then washed twice with 1X PBS to remove cell debris, before adding fresh complete DMEM media. Pictures were taken at Zero, 6, and 24-hour time points using the Olympus IX70 inverted microscope. Areas of the wounds were measured using ImageJ 1.51j8 software and percentage wound closure determined using the equation below (Boleman et al., 2012):

**Percentage wound closure % = [(wound area at zero time - wound area at X hrs) / wound area at zero time] x 100**

Where X refers to a specific time point.
2.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Analysis of differential gene expression between treated and untreated cells was performed using semi-quantitative RT-PCR in which 0.5 μg of total RNA was reverse transcribed using RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, USA) facilitated by random primers in a final volume of 20 μl, according to the manufacturer’s protocol.

The PCR reaction was performed using 1 μl cDNA template with the MyTaq DNA polymerase kit (Bioline) with GAPDH serving as an endogenous control. With the exception of cycle numbers and annealing temperatures (Table 1), PCR conditions were as follows among all tested genes; 94°C for 3 minutes, followed by cycles of (94°C for 30 seconds, annealing temperature for 30 seconds and 72°C for 45 seconds) with a final extension carried out at 72°C for 7 minutes. PCR products were analyzed on 2% agarose gel and visualization done using Gel Doc EZ System (Bio-Rad, USA). Working solution concentrations for all primers used were set to 10 pmol with the exception of GAPDH primers whose working concentration was set to 5 pmol.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’to 3’)</th>
<th>Annealing Temperature (°C)</th>
<th>Cycles</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twist-1</td>
<td>F-AGCTACGCCTTCCCTCCGCTCTG 60</td>
<td>37</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-CTCCCTTCTGGAAACAATGACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>F-CATGAGCGGCTGTCAGATAG 55</td>
<td>35</td>
<td>643</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-CTGAGTCAGGCCCTTCTGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>F-GCGACAAGAAGTATGGCTTC 58</td>
<td>30</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TGCCAACGGTCATATGTCAAGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP1</td>
<td>F-ACAGCCTGCCAGCGACTCTA 60</td>
<td>33</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-CAGGGTTTCAGCATCTGGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAX</td>
<td>F-ATGGACGCGGCTCCGGGAG 60</td>
<td>33</td>
<td>305</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TCAAGAAAACATGTACGCTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cadherin</td>
<td>F-CACTGCTCAGGACCGAGAT 60</td>
<td>30</td>
<td>416</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TAAGCCGAGTGATGGTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-catenin</td>
<td>F-ACTGGCAGCAACAGTCTTACC 61</td>
<td>30</td>
<td>1228</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TTTGAAGGACTGCTCGAATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td>F-GATGGGACTGTGGTTACTGTCA 60</td>
<td>33</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-CTCAGATCGTTGAACCTTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>F-GAACGCAGATGCGTGAATAG 60</td>
<td>33</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-CCAGAGGAGTGAATCCAGATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F-CCACCCATGGCAAATTCATGGCA 60.5</td>
<td>26</td>
<td>595</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TCTAGACGGCGTACGGTCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.9 In Silico Analysis

U2OS microarray data sets in which U2OS cells were treated with either doxorubicin or Nutlin-3 (Menendez et al., 2013) were retrieved from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) database. Microarray datasets were processed in R using Affy analysis of the Bioconductor 2.0 package (http://www.bioconductor.org/packages/release/bioc/html/affy.html) (Gautier, Cope, Bolstad, & Irizarry, 2004). Analysis was made for differentially expressed genes with p value cut offs set at either 0.01 or 0.05 and fold change cut offs set to either 2 or 3. Accession numbers and description of the various datasets used are shown in Table 2.

2.11 Statistical Analysis

All Data generated were presented as mean ± standard deviation of three independent experiments unless otherwise specified and was analyzed using GraphPad Prism 5.0 software. Multiple comparison analyses were performed using either two-way or one-way ANOVA followed by Dunnett’s post multiple comparisons test. Pairwise analyses were on the other hand performed using Prism’s multiple t tests analysis with multiple comparisons corrected for using the Holm-Sidak method.

Dose response curves and IC50 values were generated in in GraphPad Prism 5.0 using the equation;

\[(\log\text{(inhibitor)}) \text{ vs. normalized response -- Variable slope)}\].
Image J software ware was used to analyze intensities of PCR bands; these were normalized to the endogenous control, GAPDH and used to calculate relative gene expression relative presented as fold change relative to untreated cells. P-values less than 0.05 were considered significant (*p < 0.05, **p-value < 0.01, ***p-value < 0.001).

Table 2. Microarray Datasets

<table>
<thead>
<tr>
<th>Gene Expression Omnibus Sample</th>
<th>Description of U20S Cells Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSM1131226</td>
<td>No treatment repeat 1</td>
</tr>
<tr>
<td>GSM1131227</td>
<td>No treatment repeat 2</td>
</tr>
<tr>
<td>GSM1131228</td>
<td>No treatment repeat 3</td>
</tr>
<tr>
<td>GSM1131229</td>
<td>DXR-treated repeat 1</td>
</tr>
<tr>
<td>GSM1131230</td>
<td>DXR-treated repeat 2</td>
</tr>
<tr>
<td>GSM1131231</td>
<td>DXR-treated repeat 3</td>
</tr>
<tr>
<td>GSM1131232</td>
<td>DMSO-treated repeat 1</td>
</tr>
<tr>
<td>GSM1131233</td>
<td>DMSO-treated repeat 2</td>
</tr>
<tr>
<td>GSM1131234</td>
<td>DMSO-treated repeat 3</td>
</tr>
<tr>
<td>GSM1131235</td>
<td>Nutlin-3-treated repeat 1</td>
</tr>
<tr>
<td>GSM1131236</td>
<td>Nutlin-3-treated repeat 2</td>
</tr>
<tr>
<td>GSM1131237</td>
<td>Nutlin 3 treated repeat 3</td>
</tr>
</tbody>
</table>

DXR, doxorubicin; DMSO, dimethyl sulfoxid
Chapter 3. Results

3.1 U2OS Growth Characteristics

To determine their growth characteristics, U2OS cells used in this study were cultured over a period of six days and the number of viable cells per ml was determined for each consecutive day (Table 3); these results were used to construct a growth curve and to determine the doubling time of the cells. The obtained growth curve (Figure 1) exhibited a sigmoidal pattern with lag, exponential and stationary phases during which cells exhibited slow, exponential and stationary growth rates respectively. The lag phase started at 0 and lasted 2 days after which the cells grew exponentially till the fourth day. Cell growth rate then decelerated between the 4th and 5th days and finally leveled off on the 6th day. Furthermore, the number of cells increased by about seven-fold during the entire growth period and the calculated doubling time was 1.2 days (Table 4).

<table>
<thead>
<tr>
<th>Table 3. Viable cell count for U2OS growth curve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (days)</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>
**Figure 1.** U2OS growth curve as determined over a 6-day period.

**Table 4.** Determination of U2OS cells doubling time

<table>
<thead>
<tr>
<th>T*</th>
<th>Xb*</th>
<th>Xe*</th>
<th>DT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5.025</td>
<td>16.25</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*DT = T ln(2)/ln(Xe/Xb)*
3.2 MTT Assay

MTT assay was utilized to determine the effect of the various *E. foeminea* extracts on U2OS cell viability over a series of serial dilutions ranging from 31.25 µg/ml to 1000 µg/ml at 24, 48 and 72-hour time points. Generally, when treated with *E. foeminea* ethyl acetate extract, U2OS cells showed a reduction in percentage cell viability that was both dose and time dependent (**Figure 2A**). Percentage viability was shown to reduce with increasing extract concentration; significant reductions in viability were observed at concentrations of 250, 500 and 1000 µg/ml for both the 48 and 72-hour time points when compared to untreated cells. However, reduction in cell viability at the 24-hour time point showed no significance. Further still, all concentrations below 250 µg/ml showed no significant reduction in viability. Furthermore, percentage viability was observed to decline with increasing incubation time for individual extract concentrations. The greatest reduction in cell viability was observed at the extract concentration of 500 µg/ml for the 72-hour time point (***p-value < 0.001) while the least significant reduction in cell viability was observed at 250 µg/ml for the 48-hour time point. 40 µg/ml of cisplatin which was used as a positive control showed significant decreases in U2OS viability at all time points tested (**Figure 2B**).
Figure 2. Effect of *E. foeminea* ethyl acetate extract on U2OS cell viability (A). Effect of 40 µg/ml of cisplatin which was used as a positive control (B). ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. n = 3.

The *E. foeminea* ethanol extract showed a similar trend to the ethyl acetate extract with regards to its effect on U2OS cell viability. Reduction in U20S percentage viability by the ethanol extract was both dose and time dependent with percentage viability shown to reduce with both increasing extract concentration and increasing incubation time of cells with the extract (Figure 3A). Concentrations of 1000 µg/ml and 500 µg/ml significantly decreased the viability of U2OS cells at all time points with p-values of less than 0.0001. The ethanol extract at 250 µg/ml significantly reduced the viability of U2OS cells at only 48 and 72-hour time points (*** p-value < 0.001). Other significant decreases in viability were observed at extract concentrations of 125 µg/ml for both 48 and 72-hour time points (p-values 0.001 and 0.02 respectively). In addition, the extract concentration at 62.5 µg/ml also showed significant decreases in viability at 24, 48 and 72-hour time points (p-values 0.0026, 0.0020 and 0.0065 respectively). The least significant reduction
in viability was observed for the 31.25 µg/ml ethanol extract concentration at only the 48-hour time point (p-value 0.002). The greatest decline in U2OS cell viability was observed for the ethanol extract with a concentration of 500 µg/ml at the 72-hour time point. 40 µg/ml of cisplatin which was used as a positive control showed significant decreases in U2OS viability at all time points tested (Figure 3B).

![Figure 3. Effect of E. foeminea ethanol extract on U2OS cell viability (A). Effect of 40 µg/ml of cisplatin which was used as a positive control (B). ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. n = 3.](image)

U2OS cells treated with *E. foeminea* water extract similarly showed a decline in viability in a manner that was both dose and time dependent (Figure 4A). Generally, cell viability decreased with both increasing extract concentration and incubation time. All concentrations of the water extract showed significant decreases in viability at all time points except the extracts at 250 µg/ml, 62.5 µg/ml and 31.25 µg/ml for the 24-hour time point. Extract concentrations that showed the highest significant decrease in percentage viability (*** p-value < 0.001) included 62.5, 125, 250, 500 and 1000 µg/ml for both the 48 and 72-hour time points. 40 µg/ml of cisplatin which was used
as a positive control showed significant decreases in U2OS viability at all time points tested (Figure 4B).

DMSO solvent in which extracts were dissolved generally had no significant effect on U2OS cell viability (Figure 5) at all concentrations for all time points. A summary of the obtained p-values for all treatments is shown in Table 5.

**Figure 4.** Effect of *E. foeminea* water extract on U2OS cell viability (A). Effect of 40 µg/ml of cisplatin which was used as a positive control (B). ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. n = 3.
Figure 5. Effect of DMSO solvent on U2OS cell viability. Saline was used as a cisplatin solvent. n = 3.
### Table 5. Summary of MTT assay P-values obtained from 2-way ANOVA.

<table>
<thead>
<tr>
<th>Extract concentration (µg/ml)</th>
<th>Ethyl acetate extract</th>
<th>Ethanol extract</th>
<th>Water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Significance</td>
<td>P-value</td>
<td>Significance</td>
</tr>
<tr>
<td><strong>24h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>ns</td>
<td>0.1127</td>
<td>***</td>
</tr>
<tr>
<td>500</td>
<td>*</td>
<td>0.0452</td>
<td>***</td>
</tr>
<tr>
<td>250</td>
<td>ns</td>
<td>0.6516</td>
<td>ns</td>
</tr>
<tr>
<td>125</td>
<td>ns</td>
<td>&gt; 0.9999</td>
<td>ns</td>
</tr>
<tr>
<td>62.5</td>
<td>ns</td>
<td>0.9999</td>
<td>**</td>
</tr>
<tr>
<td>31.25</td>
<td>ns</td>
<td>0.9956</td>
<td>ns</td>
</tr>
<tr>
<td><strong>48h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>***</td>
<td>0.0007</td>
<td>***</td>
</tr>
<tr>
<td>500</td>
<td>**</td>
<td>0.0041</td>
<td>***</td>
</tr>
<tr>
<td>250</td>
<td>*</td>
<td>0.0259</td>
<td>***</td>
</tr>
<tr>
<td>125</td>
<td>ns</td>
<td>0.9994</td>
<td>0.0003</td>
</tr>
<tr>
<td>62.5</td>
<td>ns</td>
<td>0.98</td>
<td>**</td>
</tr>
<tr>
<td>31.25</td>
<td>ns</td>
<td>0.9276</td>
<td>*</td>
</tr>
<tr>
<td><strong>72h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>**</td>
<td>0.0026</td>
<td>***</td>
</tr>
<tr>
<td>500</td>
<td>***</td>
<td>&lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td>250</td>
<td>**</td>
<td>0.0023</td>
<td>***</td>
</tr>
<tr>
<td>125</td>
<td>ns</td>
<td>0.3794</td>
<td>ns</td>
</tr>
<tr>
<td>62.5</td>
<td>ns</td>
<td>0.863</td>
<td>*</td>
</tr>
<tr>
<td>31.25</td>
<td>ns</td>
<td>0.9977</td>
<td>ns</td>
</tr>
</tbody>
</table>
3.3 IC50

Results obtained from the MTT assay were used to determine the IC50 values for all extracts at all time points using non-linear regression analysis (Figure 6). Dose response curves for *E. foeminea* ethyl acetate, ethanol and water extracts were plotted for 24, 48 and 72-hour time points and used to generate IC50 values per extract per time point (Table 6). The least IC50 values obtained per extract were 80.353 µg/ml at 72 h, 97.499 µg/ml at 48 h and 30.761 µg/ml at 48 h respectively for ethyl acetate, ethanol and water extracts.

![Figure 6. U2OS dose-response curves for *E. foeminea* ethyl acetate (A), ethanol (B) and water (C) extracts. Percentage viabilities were normalized to a scale running from 0 to 100.](image-url)
Table 6. Summary of IC$_{50}$, hill coefficient and $R^2$ values obtained for all extract treatments including cisplatin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>IC50 ± Std.Error (µg/ml)</th>
<th>Hill Coefficient ± Std. Error</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc</td>
<td>24</td>
<td>223.872 ± 1.241</td>
<td>2.898 ± 1.644</td>
<td>0.586</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>167.109 ± 1.193</td>
<td>3.450 ± 1.649</td>
<td>0.704</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>80.353 ± 1.233</td>
<td>1.284 ± 0.352</td>
<td>0.762</td>
</tr>
<tr>
<td>EtOH</td>
<td>24</td>
<td>98.401 ± 1.573</td>
<td>0.645 ± 0.250</td>
<td>0.520</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>97.499 ± 1.188</td>
<td>1.118 ± 0.225</td>
<td>0.846</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>167.880 ± 1.129</td>
<td>1.499 ± 0.253</td>
<td>0.891</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>24</td>
<td>173.780 ± 1.589</td>
<td>0.858 ± 0.401</td>
<td>0.431</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>30.761 ± 1.400</td>
<td>0.772 ± 0.194</td>
<td>0.804</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>55.847 ± 1.348</td>
<td>0.660 ± 0.153</td>
<td>0.781</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>72</td>
<td>3.473 ± 1.106</td>
<td>2.108 ± 0.340</td>
<td>0.875</td>
</tr>
</tbody>
</table>

The least extract IC50 values obtained were chosen for further analyses; these IC50 values were thus compared to the IC50 of cisplatin when incubated with U2OS cells for 72 hours. The effect of varying concentrations of cisplatin ranging from 5 to 160 µg/ml on the viability of U2OS cells was studied; all cisplatin concentrations tested significantly reduced the viability of U2OS cells (*** p-value < 0.001) (Figure 7A). These results were used to generate a dose-response curve (Figure 7B) which were in turn utilized to calculate the IC50 of cisplatin at the 72-hour time point.

*R-squared* is a “statistical measure” that indicates how close the plotted data are to the non-linear regression model used to determine IC50 values.
(Table 6). Cisplatin IC50 (3.473 µg/ml) was over 18 times lower than that of all E. foeminea extracts tested at the 72-hour time point.

Obtained hillslope coefficients for cisplatin, ethyl acetate (72h), ethanol (48h) and water (48h) extracts were compared; apart from the water extract, all other treatments had hillslope coefficients of greater than 1 with the highest coefficient observed for cisplatin (Table 6).

![Figure 7](image)

**Figure 7.** Effect of cisplatin on viability of U2OS cells at 72-hour time point ***P < 0.001; saline solution was used as a solvent for cisplatin (A). U2OS dose-response curve for cisplatin at 72-hour time point (B). Percentage viabilities were normalized to a scale running from 0 to 100.

3.4 Cell Morphology

The effect of E. foeminea extracts on U2OS cell morphology was studied. Ethyl acetate, ethanol and water extract treatments adjusted to respective IC50 values at 72 hours showed no marked effect on cell morphology when compared to un-treated cells at all time points. Generally, U2OS cells used for this study grew as a monolayer adherent to the surface of the culture flasks; the cells
mostly possessed an elliptical shape in which they appeared wider at the center but tapered on either end (Figure 8).

Figure 8. Effect of *E. foeminea* ethyl acetate, ethanol and water extract treatments on U2OS cell morphology.

3.5 Trypan Blue Exclusion Assay

U2OS cells were incubated with *E. foeminea* ethyl acetate, ethanol and water extracts at concentrations corresponding to their respective IC50 values at the 72-hour time-point and incubation done for 24-, 48- and 72-hours. The percentage of dead cells was determined by making cell counts using the trypan blue exclusion method. When compared to untreated cells, there was no significant difference in the percentage of dead cells for all treated cells at all time points except for the ethanol extract at the 24-hour time point (*p*-value = 0.0118) (Figure 9); the ethanol extract
generally showed the highest percentage of dead cells when compared to the water and ethyl acetate extracts at all time points.

**Figure 9.** Percentage of dead cells in *E. foeminea* ethyl acetate, ethanol and water extract treatments at 24, 48 and 72-hour time points.

### 3.6 Wound Healing Assay

Wound healing assay was performed, and the percentage wound closure was determined for both 6- and 12-hour time points. At the 6-hour time point, results of percentage wound closure showed that untreated cells had the greatest wound closure, followed by cells treated with the water extract, then ethyl acetate and finally ethanol extract; at this time point, percentage wound closure for all cells treated with extract was lower than that of untreated cells, although significant decline in percentage wound closure was observed for only cells treated with ethanol extract (* p-value = 0.013). There was no significant difference in percentage wound closure at the 12-hour timepoint when treated cells were compared to untreated cells. Similar trends were observed for the rate of
wound closure with significant results obtained for only ethanol extract (* p-value = 0.023) at the 6-hour time point (Figure 10).

Figure 10. Effect of E. foeminea extracts on U2OS cell migration. Wound healing assay representative images (A). Graph of percentage wound closure (B).

3.6 RT-PCR

mRNA expression for various genes involved in either cell proliferation and apoptosis or cell migration was assessed in both treated and untreated cells using RT-PCR (Figure 11).

mRNA steady-state levels for several genes involved in either cell proliferation and apoptosis (RUNX2, B-catenin, p53, BAX and Twist-1) (Figure 12) or cell migration (RUNX2, MMP2, MMP1, N-cadherin, Twist-1 and vimentin) (Figure 13) were assessed for U2OS cells treated with either ethanol, water or ethyl acetate extracts adjusted to the least IC50 value obtained per extract. The expression of all genes tested was significantly decreased by varying folds in both the ethanol and water extracts. The ethanol extract was shown to reduce expression of the tested genes much more than the water extract. The genes whose expression was reduced the most by the ethanol extract included Twist1, p53 and MMP2 while B-catenin, RUNX2 and vimentin were the least
affected. A similar trend was observed for the ethyl acetate extract in which all tested genes were down regulated; however, apart from Twist-1, the down regulation of all other tested genes by the ethyl acetate extract showed no statistical significance when compared to the untreated cells (Table 7).

![Figure 11](image-url)  
**Figure 11.** Representative agarose gel images for all genes tested for cells treated with either ethanol or water extracts. All genes were normalized to GAPDH which was used as an endogenous control.
Figure 12. Effect of *E. foeminea* ethyl acetate (72h), ethanol (48h) and water (48h) extract treatments on expression of genes involved in cell proliferation and apoptosis. Relative expressions of tested genes in cells treated with either ethanol or water extract (upper panel) and those treated with ethyl acetate extract (lower panel).
Figure 13. Effect of *E. foeminea* ethyl acetate(72h), ethanol(48h) and water(48h) extract treatments on expression of genes involved in cell migration. Relative expressions of tested genes in cells treated with either ethanol or water extract (upper panel) and those treated with ethyl acetate extract (lower panel).
Table 7: **mRNA steady state** gene expression for markers involved in cell proliferation, apoptosis and migration shown as fold change relative to that observed in untreated cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>EtOH Fold change</th>
<th>EtOH P value</th>
<th>H2O Fold change</th>
<th>H2O P value</th>
<th>EtOAc Fold change</th>
<th>EtOAc P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twist-1</td>
<td>-19.360</td>
<td>&lt; 0.001</td>
<td>-4.870</td>
<td>&lt; 0.001</td>
<td>-6.33</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>p53</td>
<td>-16.038</td>
<td>&lt; 0.001</td>
<td>-4.166</td>
<td>&lt; 0.001</td>
<td>-1.857</td>
<td>0.035</td>
</tr>
<tr>
<td>MMP2</td>
<td>-14.657</td>
<td>&lt; 0.001</td>
<td>-4.383</td>
<td>&lt; 0.001</td>
<td>-1.994</td>
<td>0.131</td>
</tr>
<tr>
<td>MMP1</td>
<td>-7.177</td>
<td>&lt; 0.001</td>
<td>-3.658</td>
<td>&lt; 0.001</td>
<td>-1.181</td>
<td>0.220</td>
</tr>
<tr>
<td>BAX</td>
<td>-6.471</td>
<td>&lt; 0.001</td>
<td>-4.172</td>
<td>&lt; 0.001</td>
<td>-3.011</td>
<td>0.027</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>-6.329</td>
<td>&lt; 0.001</td>
<td>-2.966</td>
<td>0.001</td>
<td>-1.181</td>
<td>0.220</td>
</tr>
<tr>
<td>B-catenin</td>
<td>-5.601</td>
<td>&lt; 0.001</td>
<td>-4.503</td>
<td>&lt; 0.001</td>
<td>-1.533</td>
<td>0.299</td>
</tr>
<tr>
<td>RUNX2</td>
<td>-5.211</td>
<td>&lt; 0.001</td>
<td>-2.993</td>
<td>0.001</td>
<td>-1.918</td>
<td>0.166</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-4.189</td>
<td>&lt; 0.001</td>
<td>-3.265</td>
<td>0.001</td>
<td>-3.265</td>
<td>-</td>
</tr>
</tbody>
</table>
3.7 Effect of Doxorubicin on U2OS Gene Expression

U2OS gene expression data when treated with doxorubicin was obtained from the GEO database; analysis for the differentially expressed genes at p value cutoff of 0.01 and fold cutoff of 3 showed that a majority of the genes were significantly downregulated while a few were upregulated (Figure 14).

Figure 14. Differential gene expression in U2OS cells treated with Doxorubicin. P-value cutoff was set 0.01 while fold cutoff was set to 3.
3.8 Comparison of U2OS Gene Expression when Treated with either E. Foeminea Extracts, Doxorubicin, or Nutilin-3

U2OS gene expression datasets for both doxorubicin and nutlin-3 treatments were obtained from the GEO database and analyzed for differentially expressed genes at a p value cutoff of 0.05 and fold cutoff of 2; comparison was made to U2OS gene expression when treated with E. Foeminea extracts. Of the 9 genes examined in this study, 5 were exclusively downregulated by E. foeminea while 2 were downregulated in both E. foeminea and doxorubicin treatments only. On the other hand, 1 gene was downregulated solely by both E. Foeminea and Nutlin-3 while all the three treatments were shown to downregulate 1 gene, RUNX2. Although E. Foeminea showed no upregulation of any of the tested genes, BAX was shown to be upregulated by both doxorubicin and Nutlin-3 treatments while Twist-1 was exclusively upregulated by doxorubicin (Figure 15), (Table 8).
Table 8. Comparison of U2OS gene expression when treated with either E. foeminea, doxorubicin or Nutilin-3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Downregulated genes</th>
<th>Upregulated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E. foeminea \cap \text{Doxorubicin} \cap \text{Nutilin-3}$</td>
<td>RUNX2</td>
<td>-</td>
</tr>
<tr>
<td>$E. foeminea \cap \text{Doxorubicin}$</td>
<td>$B$-catenin, $N$-cadherin</td>
<td>-</td>
</tr>
<tr>
<td>$E. foeminea \cap \text{Nutilin-3}$</td>
<td>Twist-1</td>
<td>-</td>
</tr>
<tr>
<td>$E. foeminea$</td>
<td>BAX, MMP1, MMP2, TP53, Vimentin</td>
<td>-</td>
</tr>
<tr>
<td>$\text{Doxorubicin} \cap \text{Nutilin-3}$</td>
<td>BIRC5, CDH12, CDH18, BAX, CDH10, CDH22, CDIP1, FAT3, DCHS2, FAT4, SOX2</td>
<td>PIDD1, TP53I11, TP53I13, TP53INP1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>CBY1, CDH4, CELSR3, CDH9, CDHR1, CDHR3, CELSR1, CPED1, CTNNA1, CELSR2, CTNNB1, DACT1, DACT3, CTNND2, FAT1, FAT3, LOC100653137///CDH23, JMY, TMBIM4, TMX2-</td>
<td>MIR6513///TMBIM1, TMBIM6, CTNND1///CTNND1, TP53AIP1, TP53I13, TP53INP2, TP53BP1, XIAP, TWIST1, TWIST2</td>
</tr>
<tr>
<td>Nutilin-3</td>
<td>CDH24, CDH6, CDHR2, CBY1, CDH11, CDH13, CDH15, CELSR2, DACT1, CDH16, JMY, LINC-PINT, TWISTNB, DCHS1, XIAP</td>
<td>LOC100653137///CDH23</td>
</tr>
</tbody>
</table>
Figure 15. Venn diagrams comparing gene expression in U20S cells treated with either *E. Foeminea*, doxorubicin or nutilin-3. Downregulated genes (A) and upregulated genes (B).
Chapter 4. Discussion

4.1 U2OS Growth Characteristics

The obtained growth curve for U2OS cells used in this study showed definite lag, exponential and stationary phases (Figure 1) during which cells respectively went through a period of slow growth as they recovered from sub-culturing stress, followed by a period of exponential growth and finally transitioned into a slow growth phase as cells reached confluency (Assanga, 2013). U2OS cell growth with respect to this study thus followed a characteristic sigmoid growth pattern with a calculated doubling time of 1.2 days (Table 4). This was similar to the doubling time of 1.25 days previously reported by Rey and colleagues (Rey et al., 2009). However, U2OS doubling times as high as 2.75 day have also been reported (Al-Romaih et al., 2007). Cell doubling times have been shown to be affected by several factors such as nutrients, temperature and metabolic wastes (Mather, 1998).

4.2 MTT Assay

The effect of *E. foeminea* ethyl acetate, ethanol and water extracts on U2OS cell viability was determined using MTT assay. The assay basically involves assessing the metabolic activity of cells by quantifying the amount of purple formazan formed after cells reduce the yellow MTT tetrazolium reagent and thus providing a reflection of the amount of viable cells present (Denizot & Lang, 1986). Our results showed that *E. foeminea* ethyl acetate, ethanol and water extracts each significantly decreased U2OS percentage cell viability in a manner dependent on both extract concentration and incubation time (Figures 2, 3 and 4); generally, longer incubation times and higher extract concentrations favored the reduction of U2OS percentage cell viability. These
results are in agreement with recent findings in which both *E. foeminea* ethanol and fruit juice extracts significantly decreased the viability of MDA MB 231 breast cancer, HCT 116 colorectal cancer and normal HaCat keratinocyte cells (Maayan et al., 2017). Our results however contradicted with those of Ben-Ayre and colleagues in which they reported that *E. foeminea* boiled water extract showed no significant effect on the percentage viability of both MDA MB 231 and SKBR3 breast cancer cells (Ben-Arye et al., 2016). This disparity of results can be attributed to the difference in extraction methods employed to obtain the *E. foeminea* extract(s) used per study; it has indeed been shown that extraction procedures can have a significant influence on physical, chemical and biological properties of the final extract (De Monte et al., 2014).

DMSO solvent in which our extracts were dissolved showed no significant effect on U2OS cell viability (Figure 5), indicating that the concentrations of DMSO present in the prepared extracts did not significantly interfere with U2OS growth.

**4.3 IC50**

Based on results obtained from the MTT assay, dose response curves were plotted for all *E. foeminea* extracts tested at 24, 48 and 72-hour time points (Figure 6) and used to determine IC50 values per extract per time point (Table 6). IC0 values at the 24-hour time point generally had low $R^2$ values (< 0.6) (Table 6) thus indicating that MTT data obtained at this time point had a relatively poor fit for the non-linear regression model used for IC$_{50}$ determination in this study. $R^2$ values have however been shown to be unreliable in determining goodness of fit for non-linear regression analyses, it is hence essential to consider other parameters such as the standard error of regression to examine goodness of fit (Spiess & Neumeyer, 2010). On the other hand, IC$_{50}$ values obtained at both 48 and 72-hour time points had relatively high $R^2$ values (> 0.7) hence indicating
a better fit between the obtained MTT data at these time points and the regression model used for IC$_{50}$ determination (Kvålseth, 1985); IC$_{50}$ values at 48 and 72-hour time points were thus more reliable to use for downstream studies when compared to values obtained at the 24-hour time point.

When IC$_{50}$ values per extract were compared at each time point, results showed that *E. foeminea* ethyl acetate extract IC$_{50}$ decreased at each consecutive time point with the 24-hour time point as a reference (Table 6). On the other hand, IC$_{50}$ values of both ethanol and water extracts were at their lowest at the 48-hour time point but slightly higher at the 72-hour time point. Since IC$_{50}$ is indicative of an inhibitor’s potency, these results suggest that the potency of *E. foeminea* extracts is influenced by the incubation time; while *E. foeminea* ethyl acetate extract was most potent at the 72-hour time point, both the ethanol and water extracts showed greatest potency at the 48-hour time point. For both the ethanol and water extracts a slight reduction in cytotoxicity is hence observed after 72h of incubating the U2OS cells with the respective extract. This could be related to the doubling time of the U2OS cells; after 72h, the cells would have doubled twice producing new “unaffected” cells, which led to the observed cytotoxicity relief.

All *E. foeminea* IC$_{50}$ values obtained in this study were relatively lower than those previously reported (Maayan et al., 2017) which were about 570 µg/ml and 750 µg/ml respectively for MDA-MB-231 and HaCaT cells treated with *E. foeminea* ethanol extract for 48 hours. This suggested that in addition to the extraction method employed, potency of *E. foeminea* probably varies from one cell line to another.

In addition, the relatively large differences in IC$_{50}$ values between different extracts at the same time point could be attributed to varying degrees of polarity of the solvents used for extraction. A comparison of the lowest obtained IC$_{50}$ values per extract shows that lower IC$_{50}$ values were observed in the more polar extraction solvents including water and ethanol than in the
moderately polar ethyl acetate solvent (Sarker, Latif, & Gray, 2005). It is likely that the more polar fractions of *E. foeminea* contain greater amounts of the active inhibitory compound(s).

Cisplatin, a drug commercially used in osteosarcoma treatment (Carrle & Bielack, 2006) had a much lower IC$_{50}$ than all IC$_{50}$ values obtained for all *E. foeminea* extracts at all time points (Figure 7, Table 6). The high *E. foeminea* extract IC$_{50}$ values relative to cisplatin IC$_{50}$ can be partly explained by the fact that the *E. foeminea* extracts were tested in their crude form without purifying the active compound(s) (Rasoanaivo, Wright, Willcox, & Gilbert, 2011).

### 4.4 Hill Coefficient

Hill coefficients for all extracts were generally above 1 with the exception of the water extract (Table 6). For biological reactions, the hill coefficient is a parameter used to reflect the steepness of dose response curves; the greater the Hill coefficient, the steeper the curve and vice versa. It has been shown that Hill coefficients could further give an idea on the number of either receptors or ligands involved in inhibitor binding. A Hill coefficient of 1 is indicative of a response elicited by the binding of a single ligand to a single receptor while Hill coefficients greater than 1 could indicate the involvement of more than one ligand involved in binding a receptor or group of receptors (Prinz, 2010). Hill coefficient results from this study thus suggest that there could be a greater variety of ligand-receptor interactions in both the ethyl acetate and water extracts than in the ethanol extract. It should however be noted that although the hill coefficient can serve to reflect the number of ligands or receptors involved in inhibition, it does not indicate the type of binding (either competitive, non-competitive, ortho-, iso- or allosteric) involved (Prinz, 2010).
4.5 Cell Morphology
Various chemotherapy drugs have been shown to influence the morphology of cancer cells after treatment in-vitro. Morphological changes induced by these treatments are usually influenced by several underlying molecular pathways affected by these drugs. Thus, changes in tumor cell morphology after treatment with therapeutic agents could serve as an indication of various molecular properties such as the metabolic state or viability of tumor cells (Mirzayans, Andrais, & Murray, 2018; Baba & Cătoi, 2007). In this study, *E. foeminea* extract showed no visible outstanding morphological changes when applied to U2OS cells (Figure 8). This could suggest that *E. foeminea* extracts probably have no effect on U2OS cell morphology. However, it is also possible that due to the limited resolution of the microscope used in this study, morphological changes that could have been induced by extract treatment were undetectable. This warrants the need to examine the effect of *E. foeminea* on U2OS cell morphology using more sensitive techniques such as differential staining, in addition to fluorescent and electron microscopy techniques to study the finer structures of the cell (Alberts & Johnson, 2002; Chen, Zhao, Wu, Yao, & Zhang, 2012).

4.6 Trypan Blue Exclusion Assay
The trypan blue assay was used to determine the percentage of dead cells (those lacking an integral membrane) in all treatments by differentially staining them with trypan blue dye (Jain et al., 2018). Although the ethanol extract treatment at the 24-hour time point showed a significantly high percentage of dead cells, all other extract treatments at all time points displayed no significant difference in the percentage of dead cells when compared to untreated cells (Figure 9). The extent
by which *E. foeminea* extracts decreased U2OS percentage viability in the MTT assay was not proportional to the percentage of dead cell observed with the trypan blue exclusion assay. This disparity could be explained by the fact that the MTT assay measures cell viability as a function of cell metabolism (Pascua-Maestro et al., 2018). It is thus possible that the observed decline in U2OS cell viability as observed by MTT was not to a large extent caused by U2OS cell death. Ethanol is known to dissolve phospholipids hence increasing cell membrane permeability (Da Silveira, Golovina, Hoekstra, Rombouts, & Abee, 2003), therefore is possible that the observed higher percentage of dead cells in this treatment is due this feature.

**4.7 Scratch Wound Healing Assay**

Cell migration has been shown to be fundamental to promoting tumor progression and metastasis (Entschladen, Drell, Lang, Joseph, & Zaenker, 2004). The scratch wound healing assay was used to determine whether *E. foeminea* extract treatments affect the migratory ability of U2OS cells by measuring how fast the treated cells closed the wound created by the scratch (Liang, Park, & Guan, 2007). Results from this study show that all extract treatments reduced U2OS percentage wound closure with a statistically significant decrease observed for only the ethanol extract treatment ([Figure 10](#)). These results thus indicate that *E. foeminea* extracts reduced the rate by which U2OS cells migrated to close the wound created by the scratch at the beginning of the assay. It is thus probable that *E. foeminea* extracts negatively influence the molecular machinery involved in U2OS cell migration.
4.8 Gene Expression

(a) Comparison of U2OS gene expression between *E. foeminea*, doxorubicin and nutlin-3 treatments

The expression of several markers involved in both cell proliferation, apoptosis, and cell migration was assessed in order to study the molecular basis upon which *E. foeminea* extracts reduced both viability and migratory ability of U2OS cells. It is worthy to note that all genes whose expression levels were assessed in this study were significantly downregulated by both ethanol and water extracts (Table 7). The effect on gene expression is largely dependent on the lipophilicity of the ligand. Ligand lipophilicity determines whether the ligand directly crosses the membrane or binds to a transmembrane receptor instead and hence affecting signal transduction cascades (Bidlack & Rodriguez, 2016). Since the water extract is polar, it is lipophobic and therefore is expected to contain a ligand that binds to a membrane receptor. This could most probably be either a G protein-coupled receptor or a receptor tyrosine kinase (Gilbert, 2000).

In fact, it is not uncommon for chemotherapeutic drugs to modulate gene expression (Estevez-Garcia et al., 2015; Hui, Satkunam, Al Kaptan, Reiman, & Lai, 2006; Korn, McShane, Troendle, Rosenwald, & Simon, 2002). Doxorubicin, a common chemotherapeutic drug against osteosarcoma (Blaney, Smith, & Grem, 1993) was shown to similarly downregulate the majority of the differentially expressed genes in U2OS (Figure 14). Doxorubicin is a non-specific drug that stops replication by preventing DNA cut by topoisomerase II from being resealed. It may also increase free radical production, hence adding to its cytotoxicity. Finally, it is also known to intercalate between base pairs; inducing histone eviction from transcriptionally active chromatin (Blaney et al., 1993).
A comparison of U2OS gene expression when treated with either *E. foeminea*, doxorubicin, or Nutlin-3 was made (Figure 15, Table 8). Nutlin is an imidazoline analogue that disrupts the interaction between p53 and MDM2 resulting in growth inhibitory state called senescence. These compounds work best on tumors that contain wild type p53. Nutlin exerts its effect with minutes (Haaland et al., 2014).

Of the 9 genes tested in this study, only one gene (*RUNX2*) was significantly downregulated by all the three treatments. It is thus possible that *RUNX2* could be a potent therapeutic target in osteosarcoma treatment (J. Pratap et al., 2006). Both *B-catenin* and *N-cadherin* were downregulated by *E. foeminea* and doxorubicin but not Nutlin-3 while *Twist-1* was downregulated by both *E. foeminea* and Nutlin-3 but not doxorubicin. It’s also worthy to note that both *MMP1* and *MMP2* which are genes involved in promoting extracellular matrix remodeling (J. Pratap et al., 2006) were exclusively downregulated by *E. foeminea*; this indicates that *E. foeminea* probably reduces extracellular matrix remodeling by downregulation of MMPs thus decreasing cell migration.

(b) **Downregulation of B-catenin steady-state mRNA**

In cancer, bioactive natural compounds have been shown to affect gene expression through modulating key transcription factors and pathways involved in cancer pathology (Shanmugam et al., 2016). In osteosarcoma, one such pathway is the Wnt/B-catenin signaling pathway which enhances the transcription of several downstream WNT-responsive genes such as those involved in the remodeling of the extracellular matrix and in cell cycle regulation when B-catenin is translocated to the nucleus (Kong & Hansen, 2009). In this study, *E. foeminea* extracts downregulated *B-catenin* steady-state mRNA expression, suggesting a decline in the amounts of
B-catenin protein and thus a subsequent decrease in the expression of downstream WNT-responsive genes. A number of studies have previously shown that several compounds of natural origin have the ability to modulate the Wnt/B-catenin signaling pathway. Curcumin was shown to suppress Wnt/B-catenin signaling by activating both Oct4 and glycogen synthase kinase 3B (GSK-3B) in both human embryonic kidney 293T cells and NCCIT human embryonic carcinoma cells. Further still, curcumin mitigated cell invasion, migration and proliferation in U2OS, SaOS-2 and HOS osteosarcoma cells via Wnt/B-catenin suppression (Shanmugam et al., 2016).

(c) Downregulation of Twist-1 and RUNX2 steady-state mRNA
Both Twist-1 and RUNX2 expression has been shown to be activated by the Wnt/B-catenin signaling pathway (J. Pratap et al., 2006; Zhao, Rahman, Chen, & Shin, 2017). Triggered expression of RUNX2 by Wnt/B-catenin signaling was shown to promote the expression of several genes such as MMP1 and MMP2 that promote cell migration and metastasis in osteosarcoma cells by remodeling the extracellular matrix (J. Pratap et al., 2006; Vega et al., 2017). In addition, Twist-1 activation has been implicated in a number of cancers in which it promotes the expression of several genes involved in angiogenesis chemo-resistance, stemness and metastasis (Zhao et al., 2017). Twist-1 has been shown to promote the expression of both N-cadherin and vimentin whose expression has been associated with increased migratory and invasive ability in several cancers. It is thus possible that the downregulation of B-catenin by E. foeminea in turn leads to decreased expression of both Twist-1 and RUNX2 transcription factors which subsequently reduces the expression of their downstream targets that in reference to this study include N-cadherin, vimentin, MMP1 and MMP2.
(d) **Downregulation of \( p53 \) and **\( BAX \)** steady-state mRNA

However, \( E. \textit{foeminea} \) treatments downregulated pro-apoptotic markers, \( p53 \) and \( BAX \) as well. Since both \( Twist-1 \) and \( RUNX2 \) are inhibitors of \( p53 \) mediated apoptosis (Kleinerman, 2014; Zhao et al., 2017), it is expected that their downregulation would cause an upregulation of \( p53 \). This wasn’t the case however, since \( p53 \) expression was shown to be downregulated in \( E. \textit{foeminea} \) treatments. It is thus probable that in addition to targeting Wnt/B-catenin pathway, \( E. \textit{foeminea} \) independently targets the \( p53 \) mediated apoptotic pathway by downregulating \( p53 \) and subsequently downregulating \( BAX \). A schematic representation of the proposed pathway by which \( E. \textit{foeminea} \) crude extracts if added downregulates migration markers (\( MMP1 \), \( MMP2 \), \( N\)-cadherin and \( \textit{vimentin} \)) along with pro-apoptotic markers (\( p53 \) and \( BAX \)) is shown in **Figure 16**.

![Proposed pathway](image.png)

**Figure 16.** Proposed pathway by which \( E. \textit{foeminea} \) downregulates the steady-state mRNA expression of markers involved in cell migration and apoptosis. By downregulating the steady-state mRNA expression of both \( B\)-\textit{catenin} and \( p53 \), downstream markers of cell migration and apoptosis are respectively downregulated.
4.9 Conclusion
In conclusion, this study shows that *E. foeminea* ethyl acetate, ethanol and water extracts significantly reduced U2OS cell viability; these extracts however showed no observable effects on U2OS morphology. Further still, the ethanol extract significantly reduced U2OS cell migratory ability. In addition, all extracts downregulated the steady-state mRNA expression of key markers involved in promoting both proliferation and migration in U2OS cells. It is thus possible that *E. foeminea* might contain active compound that target pathways involved in both proliferation and migration of U2OS osteosarcoma cells.

4.10 Future Perspectives
1. It seems clear that *E. foeminea* ethyl acetate, ethanol and water extracts contain different phyto-constituents with different cytotoxicities; chemical profiling, analysis of active extracts and identification of compounds using methods such as High-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and/or gas chromatography-mass spectroscopy (GCMS) is required (Jiang, David, Tu, & Barbin, 2010).

2. The process by which *E. foeminea* downregulated pro-apoptotic markers, *p53* and *BAX* remains to be elucidated; further examination of other modes of cell death such as autophagy and necrosis is also necessary.

3. Since only one cell line was used in this study, testing the effect of *E. foeminea* extracts on other osteosarcoma cell lines in addition to a healthy cell line is key.
4. The mechanism by which *E. foeminea* downregulates *B-catenin* steady-state mRNA expression is worthy of further study since *E. foeminea* may be a likely source of therapeutic compounds targeting the Wnt/B-catenin signaling pathway in osteosarcoma.
References


https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T201710A9171016.en


