# Cytotoxic and Antibacterial Activity of an Extract from a Saudi Traditional Medicinal Plant *Equisetum Arvense*

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# EXAMINATION COMMITTEE APPROVALS FORM

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# ABSTRACT

## Cytotoxic and Antibacterial Activity of an Extract from a Saudi Traditional Medicinal Plant *Equisetum Arvense*

Salsabil Abdelrahim Aldaas

**Background:** Many ancient civilizations have used plants for medicinal purposes and indeed research has suggested that plant-derived compounds can be useful for treating many ailments, including cancer and infectious diseases. One such plant, *Equisetum arvense*, commonly known as horsetail, is a herbal plant that grows in Saudi Arabia and is traditionally used as a diuretic.

**Objective (s):** We sought to determine whether horsetail extract exhibits 1) cytotoxic activity on cell lines and 2) antibacterial activity on the bacterial strain *Escherichia coli*.

**Materials and Methods:** Using dried aerial part of the horsetail plant, a methanolic extract was prepared for screening. This extract was examined for its cytotoxic effect on the following cell lines: cervical adenocarcinoma and breast adenocarcinoma as a cancer cell model; lung fibroblast as a normal cell model; and human embryonic kidney. After 72 hours of treatment, the cells were assayed to determine the relative percentages of dead and live cells. Microscopical examination was used to give approximate percentages and a general overview of the effect on cell morphology. The LIVE⁄DEAD® Viability⁄Cytotoxicity kit was used to determine viability of cells in the population by using two dyes: the green-fluorescent calcein-AM which stains living cells, and the red-fluorescent ethidium homodimer-1 which stains dead cells. The alamarBlue® assay, based on a fluorometric/colorimetric growth indicator that detects metabolic activity, was used to establish a relative percentage of the living cells in a population

treated with the plant extract compared to untreated cells (control). To determine antibacterial activity, the disc diffusion method was used.

**Results:** Preliminary screening suggests that the horsetail extract induces death on the four tested cell lines with the greatest effect on human embryonic kidney cells followed by breast adenocarcinoma. The extract also displayed antibacterial activity at the highest concentration tested.

Future studies will focus on identifying and characterizing the active components within the horsetail extract that exert the cytotoxic and/or antibacterial activity. This may provide a new and novel therapeutic avenues for the treatment of cancer and specifically renal cell carcinoma (RCC) and urothelial cell carcinoma (UCC) patients. This also may suggest nephrotoxic effect possessed by the plant which has not been investigated by any previous studies.

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# Chapter I

# INTRODUCTION

## 1.1 Overview of medicinal plants

Plants have developed complex mechanisms to survive. They have the capacity to defend themselves from predators and inhibit other plants that compete with them for space; thus, some plants produce toxic substances, such as terpenes and alkaloids, that inhibit the growth of surrounding plants [1]. Plant-derived compounds throughout the years have been a very promising source for medicinal use; many have been used as drugs, either in their original or semi-synthetic forms [2]. High effectiveness and low side effects have always been the gold standard for drug development programs. Many pharmaceutical agents have been discovered by screening natural products from plants and other natural sources. Compared to randomly synthesized compounds, drugs derived from natural resources represent a significant segment of the pharmaceutical market [3]. Therapeutic efficacy of phytotherapy is based on the combined action of a mixture of constituents, and since many diseases are multi-factorial in nature, it provides an effective treatment strategy [3]. In addition to their effectiveness, plant-derived therapies generally have relatively preferable safety profiles. Chinese herbal medicine, for example, appears to be relatively safe with comparatively few reports of adverse reactions compared with prescription drug use [4]. Of course, adverse effects from chronic dosing of traditionally used plant based remedies are not unexpected.

Currently, around 80% of the world's population, mostly in Africa, Asia and Latin America, rely on plant-based traditional medicine for primary health care. Plants formed the basis of complicated traditional medicine practices such as Ayuverda in Indian culture, and traditional Chinese medicine (TCM) in the Chinese culture. Plant-based therapies also have a significant role in the health care system in developed countries, where it is usually referred as complementary or alternative medicine (CAM). In addition, about 25% of prescribed medicines are still derived either directly or indirectly from plants. Over the last 20 years, around 850 of the small molecules introduced as drugs were of plant origin, and over 100 are currently in clinical trials [5]. Their use has increased steadily over the last 10 years. In the USA alone, total estimated herbal sales in 1995 was $2.5 billion, while in 2005 it increased to $4.4 billion [2]. The reason for the renewed interest in natural product research may be due to the failure of many other drug discovery methods to deliver novel compounds for important therapeutic areas such as metabolic diseases , anti-infectives, and immunosuppression [6].

Much interesting research continues to demonstrate that plants are a very promising source for treatment of complicated diseases. In the most recent decade, natural products for the treatment of Alzheimer's disease have attracted much attention [7]. Also, development of neuroprotectants from traditional herbal medicine is considered to be a promising treatment for cerebral ischemia, a complex pathological process [8]. Plant derived compounds are also effective in very common health problems like hypertension, diabetes, and obesity. Recently , a number of randomized, controlled

intervention trials have shown that consumption of green tea catechins (270 mg to 1200 mg/day) may reduce body weight and fat [9]. In addition, some plant bioactive molecules have played an important role in cancer treatment.

**1.2 Significance of cancer and plant-derived drugs**

Cancer is a generic term for a large group of diseases that can affect any part of the body. The main feature of cancer is rapid abnormal growth of cells beyond their usual limits, which can invade neighbouring tissues or spread to other organs. Many cancers are fatal.

In fact, cancer is a leading cause of death worldwide and accounts for around 7 million deaths every year (around 13% of all deaths). The most common types of cancer worldwide are: lung cancer with 1.4 million deaths; stomach cancer with 740,000 deaths; liver cancer with 700,000 deaths; colorectal with 610,000 deaths; and breast cancer with 460,000 deaths yearly [10].

Cancer is usually managed by chemotherapy, radiation therapy, surgery, immunotherapy, monoclonal antibody therapy, or combinations of these methods. The treatments used varies depending on the type of cancer and location, disease stage, as well as the general health status of the patient. In general, cancer is known as a deadly disease due to its poor prognosis. For example, taken as a whole, about half of patients receiving treatment for invasive cancer (excluding carcinoma *in situ* and non-melanoma

skin cancers) die from cancer or its treatment. This illustrates the need to develop new and more effective anticancer drugs .

Natural products represent one of the most valuable sources for new drugs with substantial untapped reserves [11]. Many compounds derived from plants are currently used in cancer treatment including but not limited to, camptothecin, vincristine, etoposide and paclitaxel [1]. Many of these natural compounds have been chemically modified to render them more active, less toxic, and more soluble [12].

- Camptothecin:

Camptothecin was isolated from *Camptotheca acuminata* (tree of love), *Ophiorrhiza pumila,* and *Mapia foetida*. It is a member of the quinolinoalkaloid group (Figure 1A). It exerts its anticancer activity through inhibition of DNA topoisomerase I, generating double stranded breaks which lead to cell death.This introduced a very new mechanism into standard chemotherapy treatment after its discovery in 1966 [13] initiating much research on it and over 3000 research papers published. In its pure form, Camptothecin is very toxic prompting researchers to synthesize less toxic analogues such as Hycamtin and camptosar used for treatment of ovarian and colon cancers [12]. Irinotecan and topotecan are also camptothecin derivatives and have been shown to have significant antitumor activity. In particular, irinotecan is used for colorectal cancer while topotecan is used for ovarian cancer [1]

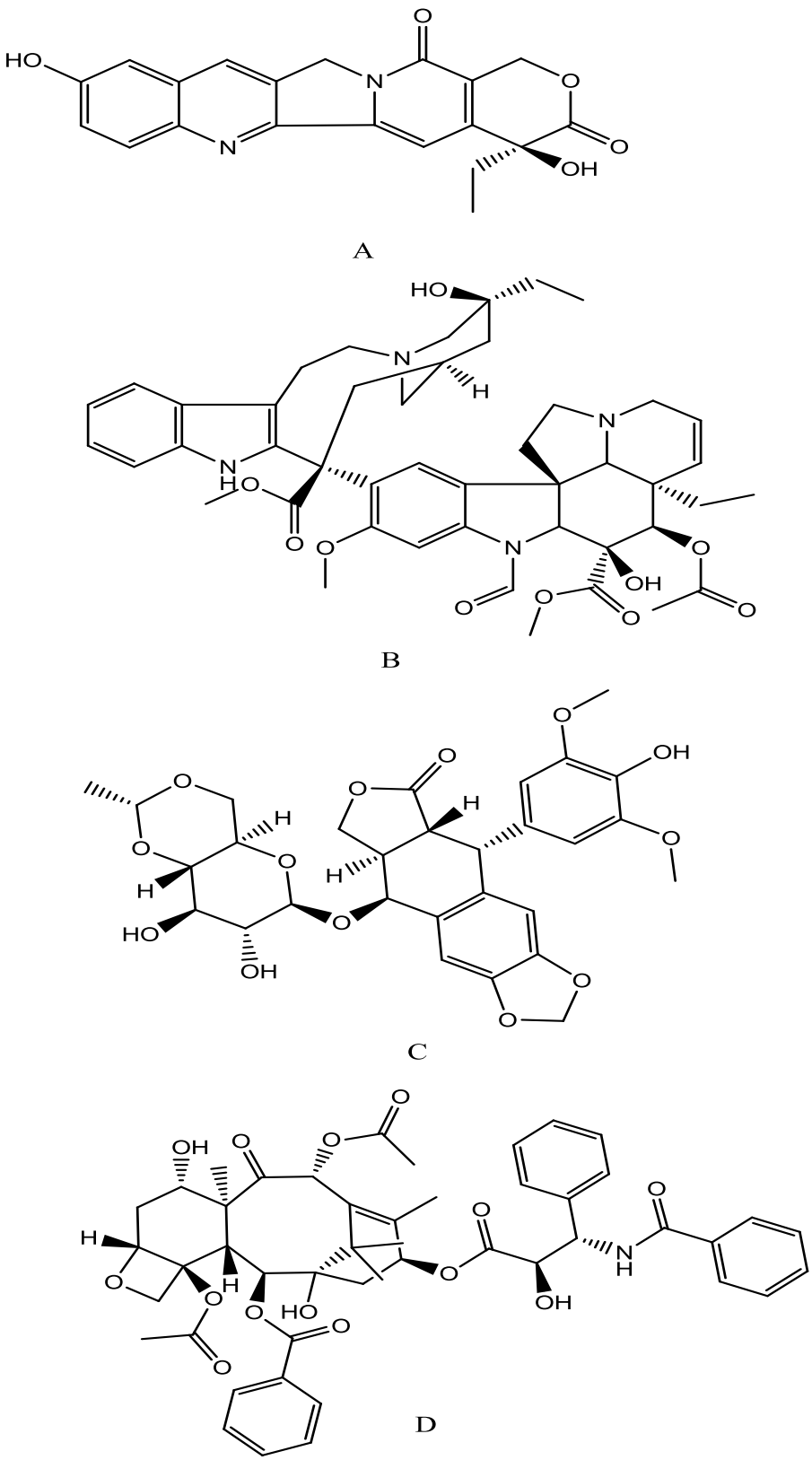



Figure 1 : chemical structure of the most important plant-derived compounds used in cancer treatment.
A: camptothecin ; B: vincristine ; C: etoposide ; D: paclitaxel

- Vincristine:

Vincristine was isolated from *Catharanthus roseus* [1]. It has significant value; since the introduction of this compound has increased the cure rates for Hodgkin's disease in addition to some types of leukemia. It has anticancer activity through inhibiting microtubule assembly [1].(see Figure 1B)

- Etoposide:

Etoposide is an epipodophyllotoxin molecule that is derived from *Podophyllum peltatum* and *Podophyllum emodi* (Figure 1C). The introduction of this bioactive molecule increased the cure rates in testicular cancer when used in combination with bleomycin and cisplatin. It exerts its activity through inhibiting topoisomerase II, leading to DNA breaks.

- Paclitaxel:

Paclitaxel is commonly known as taxol. It was first isolated from *Taxus brevifolia* (the pacific yew tree). Later on, it was also isolated from other species; like *Taxus wallichiana*. Paclitaxel stabilizes microtubules, leading to mitotic arrest [1]. Taxol was approved in 1992 to be marketed for the treatment of refractory ovarian cancer, metastatic breast and lung cancer, and Kaposi's sarcoma. A semisynthetic analogue was developed, called taxotere. Taxotere is known as a better anticancer drug than taxol [12]

### 1.3 Aim of the project

The plant *Equisetum arvense* has been used for generations in the Saudi Arabian traditional medicine for kidney problems. This study aims to evaluate the ability of the plant extract to exert cytotoxicity against various cancer cell lines including cervical adenocarcinoma, lung fibroblast, breast adenocarcinoma, and human embryonic kidney cells. Potential anti-bacterial activity against *Escherichia coli* was also evaluated.

### 1.4 *Equisetum arvense*

*Equisetum arvense*, commonly known as the field horsetail or common horsetail, is native to the northern hemisphere and is a rather bushy perennial with a rhizomatous stem formation (Figure 2). These plants either have sterile or fertile stems. After the fertile stems have wilted, sterile stems start to grow. The sterile stems are usually much taller and bushier, and are the ones used for medicinal purposes.

#### 1.4.1 Taxonomic Classification:

| | |
|---|---|
| Kingdom: | Plantae |
| Division: | Pteridophyta |
| Class: | Equisetopsida |
| Order: | Equisetales |
| Family: | Equisetaceae |
| Genus: | Equisetum |
| Species: | arvense |


Figure 2: *Equisetum arvense* (field horsetail)

1.4.2 Traditional use:

Horsetail is common in many places, including the U.S., Canada, Europe, and many parts of Asia including Iran and Turkey, in addition to the Middle East.

*Equisetum arvense* is a very well known herb in Saudi Arabia and commonly used as a traditional medicinal plant. It is used for a variety of problems, including as a diuretic, in the treatment of kidney and bladder problems, gastroenteritis, and prostate and urinary infections. It is also particularly indicated for enuresis in children. Another common use explains the derivation of the locally used name “pregnancy herb”, since this herb is used by women who fail to conceive. It is believe that this plant eliminates polyps and sacculation in the uterus. The dried aerial part of the plant is consumed as such, or with milk or any hot drinks. The plant has also been used topically to treat wounds and chilblains by dipping a piece of gauze in a water extract of the plant made by boiling the dried plant.

1.4.3 Biological evaluations of *Equisetum arvense*

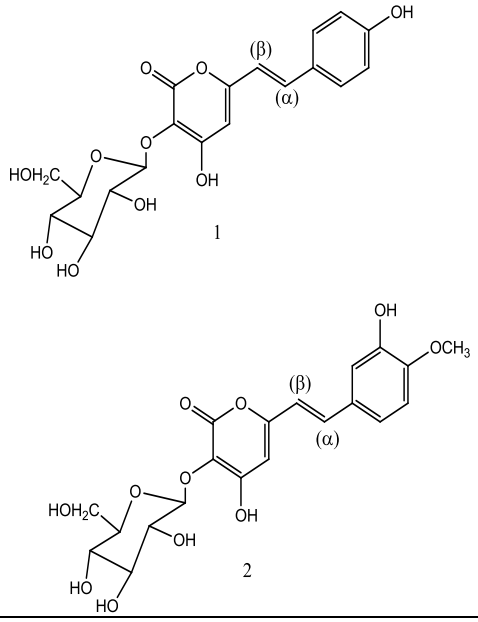
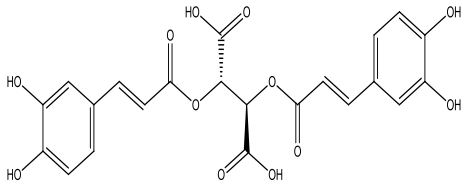
A number of studies have been published describing different biological effects of this plant. Horsetail preparations were proved to have effects on diuresis, are antioxidant, vasorelaxant, antinociceptive, anti-inflammatory and possess germination inhibitory activity, as well as many other effects [14].

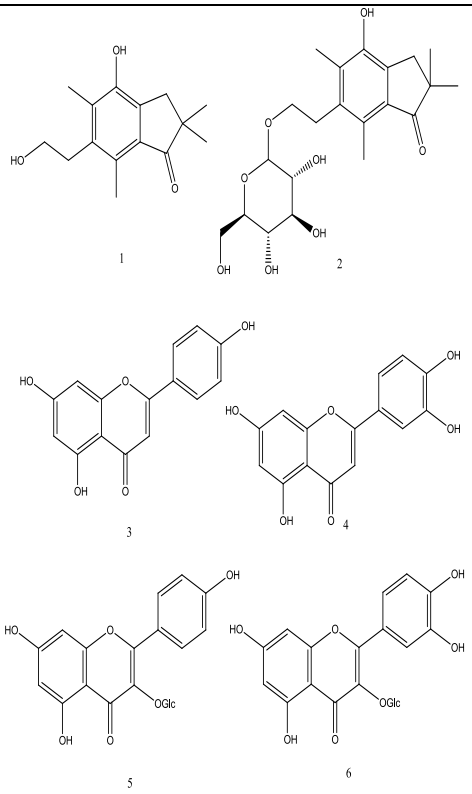
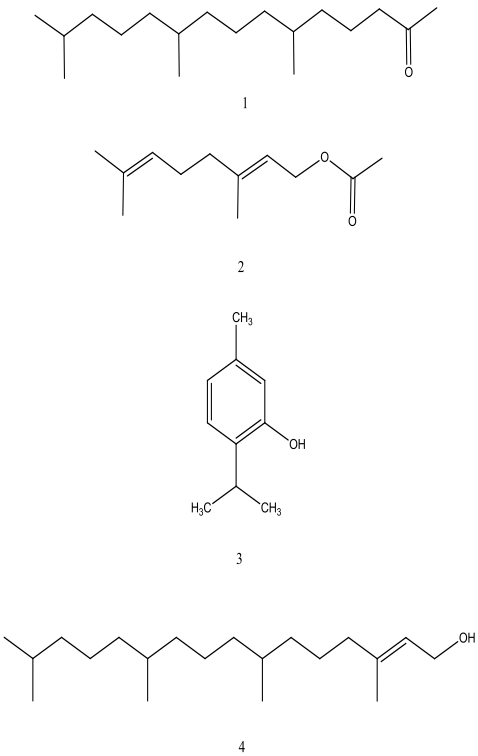
For example , aqueous extracts of the aerial part of *Equisetum arvense* showed a dose-dependent inhibition of thrombin and ADP-induced platelet aggregation in a study on

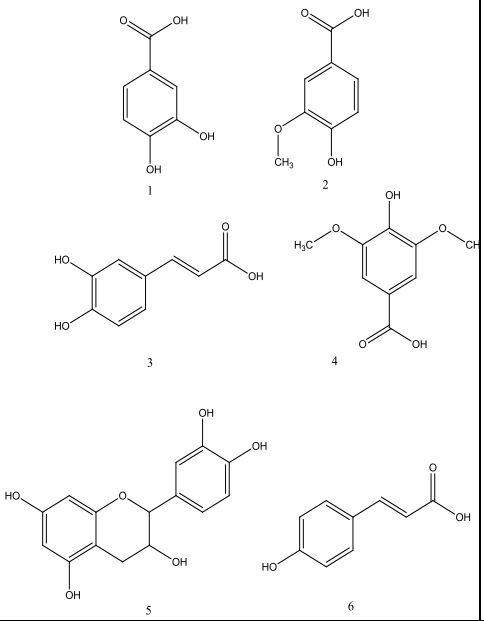
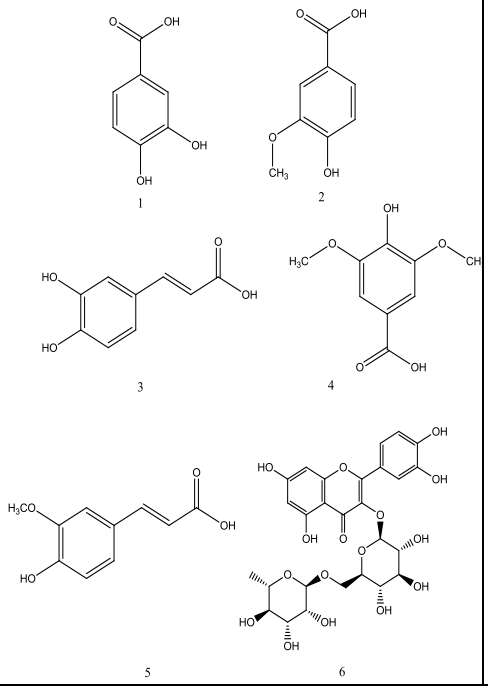
some Moroccan medicinal plants [15]. Trouillasa et al [16] demonstrated the antioxidant and anti-inflammatory capabilities of the water-soluble fractions of hydro-alcoholic extracts of horsetail in France. The antioxidant properties were evaluated by the electron spin resonance (ESR) method in order to visualize inhibition of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) superoxide and hydroxyl radicals. Anti-inflammatory activities were measured by evaluating inhibition of lipoxygenase activity. Another study was conducted to test anti-oxidative activity of n-butanol, ethyl acetate, and water extracts of the aerial parts of the plant. As in the previous study, it was tested by measuring their ability to scavenge stable DPPH and reactive hydroxyl radicals by ESR spectroscopy. The results showed that this activity was dependent on the type and concentration of the extracts, where the highest activity was obtained by the n-butanol extract. The activity increased dose-dependently at concentrations ranging from 0.5 to 2.5 mg/mL. This was correlated with the total phenolic content which was determined by the Folin-Ciocalteu method [17]. Total phenolic content was shown to be in the following order: n-butanol > methanol> ethyl acetate> water> chloroform> petroleum ether (with no phenolic content) extract [18].

Moreover, some bioactive molecules were isolated from *Equisetum arvense* and tested for their biological activity (see Table 1). For instance dicaffeoyl-meso-tartaric acid was isolated from the plant and showed a vasorelaxant activity in isolated rat aorta strips [19]. Furthermore, hepatoprotective activity and superoxide scavenging effects were shown in two compounds isolated from fractionation of the methanol extract of the plant; onitin (phenolic petrosins) and luteolin (flavonoids). Hepatoprotective activities

were performed on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells [20].

| Type of *E. arvense* preparation | compound | Structure | Biological activity | Reference |
|---|---|---|---|---|
| Methanolic extract of lyophilized rhizome | Phenolics : styrylpyrone glucosides<br><br>1-deoxyequisetumpyrone (3,4-hydroxy-6-(4'-hydroxy-E-styryl)-2-pyron-3-O-fl-D-glucopyranoside)<br><br>2-4'-O methylequisetumpyrone (3,4-hydroxy-6-(3'-hydroxy-4'-methoxy-E-styryl)-2-pyron-3-O-fl-D-glucopyranoside) |  <br>1<br>2 | | [21] |
| Not determined | dicaffeoyl-meso-tartaric acid |  | Vasorelaxant | [19] |
| Water soluble fractions of hydro-alcoholic horsetail | Phenolic compounds, tannins | | antioxidant, and the anti-inflammatory | [16] |
| Methanol extract of the aerial parts | phenolic petrosins:<br>1-onitin<br>2-onitin-9-O-glucoside<br><br>Flavonoids: | | - Hepatoprotective activity (1 and 4 only)<br>- superoxide scavenging | [20] |

| | | | | |
|---|---|---|---|---|
| | 3-apigenin<br>4- luteolin<br>5-kaempferol-3-O-glucoside<br>6-quercetin-3-O-glucoside |  | effects<br>(1 and 4 only) | |
| the volatile constituents of the sterile stems | Major constituents:<br><br>1-Hexahydrofarnesyl acetone (18.34%)<br><br>2-cis-geranyl acetone (13.74%)<br><br>3-thymol (12.09%)<br><br>4-trans-phytol (10.06%) |  | Antimicrobial | [14] |
| Not determined | Minerals:<br>-silica(10%) | -Si | -Diuretic | [17] |

| | | | | |
|---|---|---|---|---|
| | -calcium<br>-magnesium<br>-selenium<br>-iron<br>-potassium<br>-zinc | -Ca<br>-Mg<br>-Se<br>-Fe<br>-K<br>-Zn | -for osteoporosis (Silicon) | |
| ethyl acetate extract | 1-protocatechuic acid<br>2-vanillic acid<br>3-caffeic acid<br>4-syringic acid<br>5- (-)-epicatechin<br>6-p-coumaric acid |  | -antioxidative activity<br>-antibacterial (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*) none for *Escherichia coli* | [17] |
| n-butanol | 1-protocatechuic acid<br>2- vanillic acid<br>3-caffeic acid<br>4-syringic acid<br>5- ferulic acid<br>6- rutin |  | -antioxidative activity<br>-bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus cereus*) none for *E. coli* | [17] |

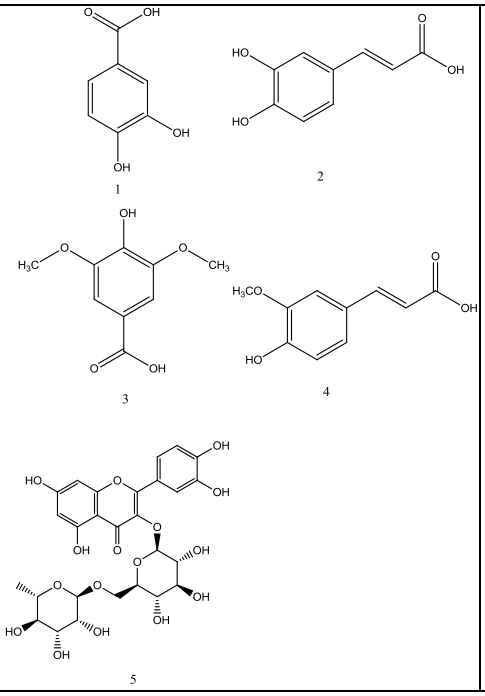
| | | | | |
|---|---|---|---|---|
| water extracts | 1-protocatechuic acid<br>2-caffeic acid<br>3- syringic acid<br>4-ferulic acid<br>5-rutin |  | -antioxidative activity<br>-Very weak antimicrobial against<br>*Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus cereus*<br>*none for E. coli* | [17] |

Table 1: Bioactive molecules identified/isolated from different extract preparations of *Equisetum arvense* with their chemical structures and biological activity.

## 1.4.4 Cytotoxic activity

Trouillasa et al [16] examined antiproliferative capabilities of the water-soluble fractions of hydroalcoholic extracts of 16 plants found in the Limousin countryside in France that are used in popular medicines. One of these was horsetail. The effects of the plant extracts on the proliferation of melanoma B16 cells were tested. At a concentration of > 0.5mg/ml, *Equisetum arvense* extract showed a significant antiproliferative effect [16]. The same study also showed antioxidant and anti-inflammatory activities. This is important because previous studies had suggested that compounds with antioxidant or anti-inflammatory activities, especially phenolic compounds, inhibit tumor promotion and cell proliferation [16].

Furthermore, in 2010 a study investigated the antioxidative and antiproliferative activity of horsetail (*Equisetum arvense*) aerial part extracts using many different solvents for extraction [18]. Antiproliferative activity was measured using the sulforhodamine B colorimetric assay on the human cancer cell lines: HeLa (human cervix epidermoid tumor), HT-29 (Human colon adenocarcinoma grade II cell line), and MCF7 (human breast adenocarcinoma cell line). The results indicate that n-butanol, methanol, ethyl acetate, and water extracts had significant peroxyl radical scavenging activity. Extracts also inhibited cell growth; this was dependent on cell line, type of extract, and extract concentration. Ethyl acetate extract exhibited the most prominent antiproliferative effect, without inducing any cell growth stimulation on human tumor cell lines. HeLa cells were found to be the most sensitive to all extracts. Antiproliferative effects on the HeLa cell line was significant using ethyl acetate, chloroform, and petroleum ether extracts in the concentration range of 0.125 to 1 mg/mL. On MCF-7 cells, growth was significantly decreased with all extracts, except n-butanol. Ethyl acetate and petroleum ether extracts exhibited a significant antiproliferative effect on the HT-29 cell line. It is worth mentioning that growth stimulation was observed in HeLa and HT-29 cell lines using a lower concentration range for all extracts, except ethyl acetate [18].

A water extract from sterile stems of *Equisetum arvense* was also tested on the human leukemic U937 cell line [22]. It showed a dose dependent cytotoxic effect. It has been suggested that this was due to apoptosis, considering the observed DNA fragmentation, externalization of phosphatidylserine, and the collapse of the mitochondrial transmembrane potential in cells cultured for 48 h with the herb extract [22].

1.4.5 Antimicrobial activity of *Equisetum arvense*

As early as the 1960's a study showed that the oil of *E. arvense* exhibited an antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and certain mycobacteria [23, 24].

Further investigation was carried out by Radulovic et al in 2006 [14] who reported that the composition of the 1:10 dilution of the essential oil obtained by hydrodistillation of aerial parts of *Equisetum arvense* possesses a broad spectrum of a strong antimicrobial activity against all tested strains of bacteria: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella enteritidis*; and fungi: *Aspergillus niger* and *Candida albicans* using a disk diffusion method for the evaluation.

In 2008, a study was conducted to screen the antibacterial activities of an extract of the whole plant of *Equisetum arvense* in addition to thirty five other plants in the Azarbaijan area of Iran [25]. The plant was used there for diuresis and osteoporosis in traditional medicine. The extracts were examined for their antibacterial activities against some gram-negative strains such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphi*, and *Serratia marcescens*, it was also tested against gram-positive strains of *Staphylococcus aureus, Micrococcus luteus, Staphylococcus epidermidis, Streptococcus pneumoniae* and *Bacillus cereus [25]*. The filter paper disc diffusion method, as well as broth serial dilution technique, was applied to screen the antibacterial efficacy of the extracts and determination of minimum inhibitory concentration (MIC) values. An ethyl acetate extract of *Equisetum arvense* showed activity against *Serratia marcescens*,

*Staphylococcus aureus*, *Staphylococcus epidermidis* (inhibition zone diameter = 11,9,10 mm respectively, MIC=2.5 mg/ml ), while a choloroform extract showed activity against *Staphylococcus aureus* and *Bacillus cereus* (inhibition zone diameter=9 each, MIC=2.5 mg/ml ) although no activity against *Escherechia coli* was observed [25].

Recently, among 14 Canadian medicinal plants, an ethanolic extract of the leaves of *Equisetum arvense* showed weak or no antimicrobial activity against the *Neisseria gonorrhoeae* isolates (evaluated by agar dilution method) with the minimum inhibitory concentration of 512 µg/mL [26].

# Chapter II

# EXPERIMENTAL

## 2.1 Chemicals and Materials

Table 2 lists chemicals and materials that were used in this study along with their detailed information:

| **Chemicals/Materials** | **Description** | **Company** |
|---|---|---|
| Agar | Lurila Bertani agar medium | SIGMA-ALDRICH |
| Ampicillin | 100 mg /mL | |
| Alamar blue | | Invitrogen |
| Calcein, AM | Calcein AM ,40 µL, 4 mM in anhydrous DMSO | Invitrogen |
| Cell lines | Human Cervical Adenocarcinoma (HeLa )<br>Human Breast Adenocarcinoma (MCF7 )<br>Human Embryonic Kidney (293T/17)<br>Human Lung Fibroblast (WI-38) | ATCC company |
| DMSO | Dimethy sulfoxide, 500 mL, certified ACS | Fisher chemical |
| Ethidium homodimer-1 | Ethidium homodimer-1, 200 µL, 2 mM in DMSO/H2O 1:4 (v/v) | Invitrogen |
| *Escherechia coli* | E. coli GC5™ competent cells | SIGMA-ALDRICH |
| FBS | Fetal Bovine Serum , heat inactivated | SIGMA-ALDRICH |
| Filter paper discs | 8 mm⦸ was cut from qualitative grade 3, 6 µm, 70 mm⦸. | Whatman |
| Flask | 50 mL, stopper No.1. | KIMAX Kimble |
| Glass vials | 20mL disposable scintillation vials | |
| Kanamycin | 50 mg/ml | |
| LB broth | Difco Luria-Bertani Broth | Miller |
| Membrane filters | 0.22 µm GV | Millipore corp. |

| | | |
|---|---|---|
| Methanol | Methanol HPLC Grade, 0.2 µ, filtred , meet ACS specifications | Fisher Scientific |
| PBS | Phosphate Buffered Saline, 1.0 M (for the experimental use, this was further diluted 10 times (10X) | SIGMA-ALDRICH |
| Petri dish | 100x 15 mm standard, polystyrene , sterile | Fisherbrand |
| Plant material | Dried aerial parts of *Equisetum arvense* were identified and provided by Abdelhamid Emwas. Ref. email: abdelhamid.emwas@kaust.edu.sa | |
| Tubes | 5 ml Polystyrene round-bottom tube, 12 x 75 mm style | BD Falcon |
| Trypsin | 2.5g porcine trypsin per litre in Hank's Balanced Salt Solution (HBSS). | SIGMA-ALDRICH |
| 6-well plate | polystyrene 6 well cell culture cluster, flat bottom with lid | Corning Inc. |
| 96-well plate | 96 well cell culture , cluster, round bottom with lid | Corning Inc. |

Table 2: List of chemicals and materials used in the study, with their description and detailed information.

### 2.2 Instrumentation and equipment

Table 3 lists instruments and equipment used in the study, with their detailed information:

| **Instrument / Equipment** | **Description** | **Company** |
|---|---|---|
| FACS | Fluorescence-Activated Cell Sorter, BD FACS Canto II | BD |
| Magnetic stirring bars | Cylindrical , 4 mm long | Fisher Scientific |
| Sonicator | Ultrasonic homogenizer model 150/vt | (biologics inc.) |
| Spectrophotometer | xMark microplate spectrophotometer | BIO-RAD |
| Stirrer | model SP 131820-33 | Thermoscientific |

Table 3: List of Instrumentation and equipment used in the study, with their description and detailed information.

## 2.3 Methods

### 2.3.1 Plant extraction:

The dried aerial part of *Equisetum arvense* was soaked in 100% methanol (by the ratio 1g: 50 mL, dried plant:methanol), and stirred vigorously (150 RPM) at room temperature for 4 days. The mixture was then sonicated for 2 hours (using power =40, pulse= 10). In order to eliminate the insoluble material, the mixture was filtered through a 6 µm pore size with 70 mm diameter filter paper. It was further filtered using a 0.22 µm membrane filter to remove finer particulate matter. The filtrate was then air dried and transferred to a pre-weighed (20 ml) glass vial. The vial containing the dried content was re-weighed and the extract weight calculated.

For use in the first cytotoxicity assay; 5 mg of the dried extract was dissolved in 10 mL water and vortexed. Thus, a stock solution of 500 µg/mL was obtained.

For use in the alamar blue assay; 1 mg of the dried extract was suspended in 1mL medium and vortexed vigorously. Thus, a stock solution of 1 mg/mL was obtained.

### 2.3.2 *In vitro* cytotoxic activity assay:

All media and cell preparations were done in a biosafety cabinet using sterile techniques. A 1 L bottle of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 was used. 100 ml was removed and replaced with 100 ml Fetal Bovine Serum (FBS) to obtain a 10% FBS Medium (v/v).

To prepare the cells, the vials of the 4 cell lines were kept in -135 degree Celsius. Cells were then allowed to thaw. Four centrifuge tubes containing 4 mL of medium with 10% FBS were prepared. For each cell line, 1 mL of medium was added to the cell vial, mixed well, then transferred to one of the previously prepared centrifuge tubes, and mixed again. Centrifugation was performed at 1200 xg for 3 mins at 4 degrees Celsius. After that, 3 mL of the supernatant were discarded from each tube, and the cell pellets that were formed were re-suspended in the remaining solution. Each suspension was then transferred to a culture flask, and 5 mL of medium containing 10% FBS were added to each flask, mixed well and incubated at 37 degrees Celsius for 3 days to allow for growth.

To transfer a portion of the cells for the assay; the media was aspirated from each cell culture flask, and the cells washed with around 3 mL phosphate buffered saline (PBS) to clear any remaining media. To detach the cells from the flask, they were incubated with 2 mL trypsin for I min. Cells then were resuspended with fresh 5 mL media (10% FBS).

For each cell line, 0.5 mL of the cell suspension was transferred to each well in a 6-well plate; four wells containing 0.5 mL suspension were transferred in for each cell line. Subsequently 2.5 ml of 10% FBS medium was added to each well. The plates were incubated at 37 degrees Celsius to allow for growth for 24 hours.

To prepare the cells for the cytotoxicity assay; after incubation, medium was aspirated, the cells were washed with PBS, incubated with 200µL trypsin, and resuspended with 1 mL fresh medium (10% FBS).

Test media were prepared as follows: for each cell line, four wells from a 6-well plate were used as previously described. Test sample final concentrations (C2) were determined using C1V1=C2V2 equation, where final volumes (V2) were aimed to be 3 mL overall well content (C1: concentration of the initial test sample , V1: initial volume added of test sample ).

- The first well was reserved as a negative control well. The well was filled with media up to 3 mL.
- The second well was prepared to test the effect of 20 µg/mL of the plant extract on the corresponding cell line. Thus, 120 µL of the previously prepared stock solution (500 µg/mL) were transferred to the well, which then was filled with medium up to 3 mL.
- The third well was prepared to test the effect of 50 µg/mL of the plant extract on the corresponding cell line. Thus, 300 µL of the previously prepared stock solution (500 µg/mL) were transferred to the well, which then was filled with medium up to 3 mL.
- The fourth well was used as a positive control to induce cytotoxicity; where the plant extract was replaced with 300 µL of DMSO (50%).

After mixing properly, the cells were transferred from the biosafety cabinet to the incubator (37ºC) for 72 hours.

To visualize the effect of the plant extract on different cell lines after the 72 hours incubation; the test samples were examined under an inverted microscope.

To obtain quantitative data, the LIVE/DEAD® Viability/Cytotoxicity kit was used. The assay is a two-color assay that determines viability of cells in a population based on plasma membrane integrity and esterase activity. Green fluorescent calcein-AM is used to indicate intracellular esterase activity, and red-fluorescent ethidium homodimer-1 is used to indicate loss of plasma membrane integrity. Thus, calcein-AM and ethidium homodimer-1 stain live and dead cells respectively.

As per the kit protocol, calcein-AM was diluted 80-fold in DMSO to make a 50 µM working solution (i.e., we added 2 µL of the stain to 158 µL DMSO).

For this assay, a 1 mL suspension of cells with 0.1 to 5 × $10^6$ cells/mL is required. Thus, for the purpose of counting cells, a device called a hemocytometer was used. 8 µL of the cell suspension were loaded and the cells were counted under an inverted microscope. The concentration of the cell suspension was calculated as follows:

1. The number of cells from a sixteen quadrant area was counted, this was repeated for different areas, and the average was calculated.
2. The average was multiplied by 2.
3. Because each quadrant contained 1 X $10^{-4}$ mL, the value was then multiplied by 10,000 to get the concentration of the cell suspension in cells/mL.

The concentration of the cell lines used were: (HeLa=1,600,000 cells/mL, H.B.A= 1,500,000 cells/mL, H.E.K=1,700,000 cells/mL), which fit within the accepted range of cell concentrations for the assay.

For each cell line, seven polystyrene round-bottom tube (5 mL) were prepared as follows:

1. As a negative control; from previously prepared cells, a total of 1 mL of media with almost complete viable cells was prepared for each cell type. The tube was kept unstained.
2. As a live cells control; from previously prepared cells, a total of 1 mL of media with almost complete viable cells was prepared for each cell type. It was then stained only with 2 µL of 50 µM calcein AM working solution.
3. As a dead cells control; from previously prepared cells, a total of 1 mL of media with almost complete viable cells was prepared for each tissue culture type. For the purpose of obtaining complete killing of the cells; 2 mL of methanol (70% final concentration) was added for 30 min . The cells were collected by centrifuging for 3 min at 1300 rpm, and then resuspended in 1 ml medium. It was then stained only with 4 µL of the 2 mM ethidium homodimer-1 stock.
4. The content of the untreated well in the 6-well plate was fully transferred to a tube. Trypsinization by 300 µL trypsin and addition of 1 mL medium was conducted to detach any remaining cells which were then washed, the tube was centrifugated for 3 min, and the supernatant was removed and substituted with 2 mL fresh media. The tube content was then double stained with 4 µL of 50 µM calcein AM working solution, and 8 µL of the 2 mM ethidium homodimer-1 stock. (The dyes are ideally added in the ratio of 2µL calcein AM working solution for

each mL suspension, and 4µL ethidium homodimer-1 stock for each mL suspension).

5. The content of the 20 µg/mL treated well in the 6-well plate was fully transferred. Trypsinization by 300 µL trypsin and addition of 1 mL medium was conducted to detach any remaining cells and wash them for collection, the tube was centrifuged for 3 min and the media was removed and substituted with 2 mL fresh media. The tube content was then double-stained with 4 µL of 50 µM calcein AM working solution and 8 µL of the 2 mM ethidium homodimer-1 stock.
6. The content of the 50 µg/mL treated well in the 6-well plate was transferred to a tube. Trypsinization by 300 µL trypsin and addition of 1 mL medium was conducted to detach any remaining cells and wash them for collection, the tube was centrifuged for 3 min and the media was removed and substituted with 2 mL fresh media. The tube content was then double stained with 4 µL calcein-AM and 8 µL of the 2 mM ethidium homodimer-1 stock.
7. The content of the DMSO treated well in the 6-well plate was transferred to a tube. Trypsinization by 300 µL trypsin and addition of 1 mL medium was conducted to detach any remaining cells and wash them for collection, the tube was centrifuged for 3 min and the media was removed and substituted with 2 mL fresh media. The tube content was then double-stained with 4 µL of 50 µM calcein AM working solution and 8 µL of the 2 mM ethidium homodimer-1 stock.

All tubes were vortexed to mix well and kept for 15 minute in a dark area. After the incubation period, 1 mL suspension in each tube was taken directly to be analyzed in a Fluorescence-Activated Cell Sorting (FACS) machine.

2.3.3 Alamar blue assay:

In order to establish confirm the cytotoxicity data; the alamarBlue® Assay was used. This assay depends on a fluorometric/colorimetric growth indicator based on detection of metabolic activity of living cells, and consequently cytotoxicity can be determined.

Cells were plated as previously described, except that they were distributed as 200 µL in each well in a 96-well plate, with each cell line occupying 15 wells. Cells were kept in an incubator to allow for growth.

For this assay, the acceptable number of cells for analysis is 100,000 cells/well (each well contain 200µL). A hemocytometer was used for counting. Eight µL of the cell suspension were loaded. The cells were counted under an inverted microscope and the concentration of the cell suspension was calculated as described previously.

The concentration of the cell lines used were:

- HeLa: 4,000,000 cells/mL = 800,000 cells/well. Thus, it was further diluted 8 times to obtain the acceptable number for analysis.
- H.B.A: 1,200,000 cells/mL = 240,000 cells/well. Thus, it was further diluted 2.5 times to obtain the acceptable number for analysis.

- H.E.K: 8,000,000 cells/mL = 1,600,000 cells/well. Thus, it was further diluted 16 times to obtain the acceptable number for analysis.
- H.L.F: 480,000 cells/mL = 96,000 cells/well. This is an acceptable number for analysis.

Two hundred µL of the new dilution was pipetted into each of the wells on the 96-well plate. The plate was then incubated (37ºC) for 24 hours.

For test samples, a wider range of concentrations of the plant extract were used; 20µg/mL (which was prepared by 60 µL of the 1mg/mL stock solution and filled up to 3 ml medium), 50µg/mL (which was prepared by 150 µL of the 1mg/mL stock solution and filled up to 3 ml medium), and 100µg/mL (which was prepared by 300 µL of the 1mg/mL stock solution and filled up to 3 ml medium). The same experiment was then repeated later for 10µg/mL (which was prepared by using 30 µL of the 1mg/mL stock solution and fill it up to 3 ml medium).

In the 96-well plate, all the media was then aspirated from each well, and 200 µL of each concentration of the extract (n=3) was pipetted into each well. For the control, 200 µL of 50% DMSO was used (stock= 15 ml DMSO:15 ml water). The inoculated plate was then incubated (37ºC) for 48 hours.

At that point, 20µL of the stain was added for each well. Readings were obtained at different time points. The data shown were obtained 2.5 hours after staining.

2.3.4 Antibiotic susceptibility testing:

The in-vitro antimicrobial activity of the *Equisetum arvense* methanol extract was tested against a laboratory control strain Gram-negative bacteria, *Escherichia coli ;* since this is one of the best studied prokaryotic model organisms, and an important species in microbiology because it can be grown easily.

The disk diffusion method was used, which is a highly recommended method for routine assessment of preliminary antimicrobial screening.

To prepare bacteria for the assay; 25 g of Difco LB Broth was dissolve in 1 L of purified water, mixed thoroughly, and autoclaved at 121 degree Celsius for 15 mins. After preparing LB Broth, 1 vial of E. coli (GC5™ Competent Cells supplied by Sigma Aldrich) was thawed, and 1 mL of LB broth was added with good mixing. This was placed on agar plates as 250 µL for each plate and spread well on the surface.

Filter paper discs (8 mm in diameter) were previously impregnated for one hour with 50, 100 µg/ml and 1, 500, or 1000 mg/ml of a plant crude extract dissolved in deionized water were placed on inoculated plates directly. After standing at room temperature for 2 h, the plates were incubated at 37°C for 24 h for bacteria to grow. Standard disks of ampicillin (100*m*g/ml), and kanamycin (50*m*g/ml) were individually used as positive controls. (2-4 trials were conducted for each concentration).

The assessment of antibacterial activity was by observing of the diameter of inhibition zone formed around the disc.

# Chapter III

# RESULTS AND DISCUSSION

## 3.1 Cytotoxicity assay

Limited research has been performed to evaluate the cytotoxic effect of *Equisetum arvense* on cell lines; however, all of them showed a significant effect on cancer cell lines. As mentioned earlier, hydroalcoholic extract of the plant showed significant antiproliferative effect on melanoma cells. While n-butanol, ethyl acetate, chloroform, petroleum ether, water, and methanol extract of the plant showed significant antiproliferative effects on MCF 7, HT-29, U937, and HeLa cancer cell lines.

We aimed to evaluate and compare the cytotoxic effect of methanolic extract of horsetail on human embryonic kidney, and the cervical and breast cancer cells (HeLa, and MCF 7 respectively). We also compared the effectiveness to a normal cell (lung fibroblast).

The dried aerial part of *Equisetum arvense* was extracted with methanol and tested against HeLa, MCF7, 293T/17, and WI-38 cell lines, using different concentrations of the extract. After 72 hours of treatment, microscopic examination showed that the extract

seemed to have cytotoxic effects on cervical, breast, and kidney cell lines (Figure 3).

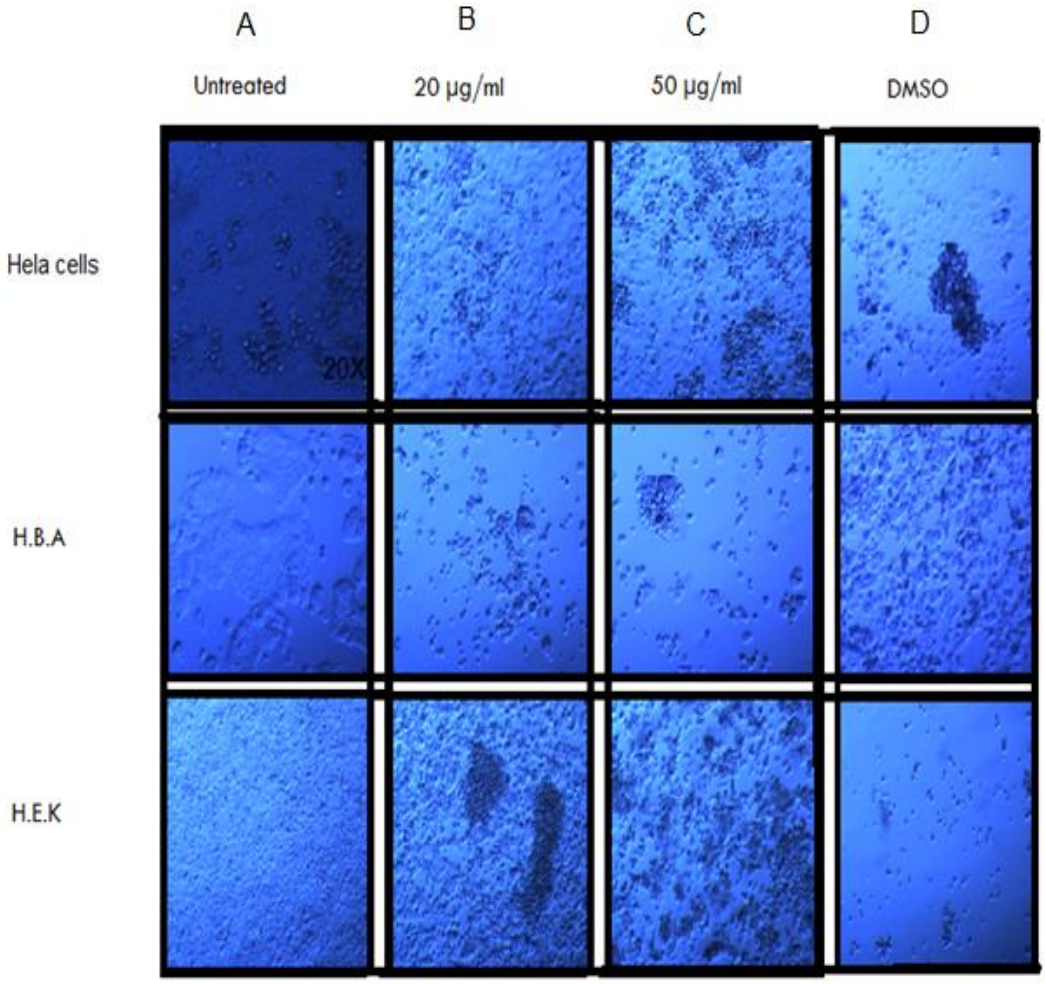



Figure 3: Cytotoxic effect on human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (H.B.A), and human embryonic kidney (H.E.K) cell lines. (A): control (untreated) and cytotoxic effect of (B): 20 µg/mL of *Equisetum arvense* methanol extract , (C): 50 µg/mL of *Equisetum arvense* methanol extract , and (D): DMSO 5%.

The extent of toxicity varied depending on the cell type and the concentration of extract used. Compared to untreated cells, the plant extract had a profound cytotoxic effect on the breast cancer cell line. This effect was concentration-dependent, where 50 µg/mL had a larger effect than 20 µg/mL. However, both concentrations were more cytotoxic than the positive control used, DMSO (5%). A promising cytotoxicity was also observed

on the embryonic kidney cell line, again, 50 µg/mL showed more activity than 20 µg/mL, however DMSO was more cytotoxic in this cell line. On HeLa cells, only a very slight difference was observed when extract-treated cells were compared to untreated cells. In addition, no difference was observed between the two concentrations of the plant extract diluted in water. This does not conflict with the principal finding by Cetojevic-Simin et al. [18] that states that methanolic extract of the plant possess significant antiproliferative effect on Hela cells; since this effect was obtained only by higher concentrations (within the range of 0.125-1 mg/mL), while at lower concentrations, slight growth stimulation was observed.

A more quantitative method was used to obtain the percentages of dead, living, or dying cells. Cells were stained with a dead cell selective stain and a live cell selective stain for 15 min, and then the degree of staining was detected using a FACS (fluorescently-activated cell sorting) machine. The outcome is shown in Figure 4.

As the histogram in Figure 5 shows, in HeLa cells, compared to untreated cells, the number of dead cells did not increase significantly when treated with horsetail extract. This is consistent with the microscopic examination data. However, the percentage of living cells decreased more than 50%. Thus the cells were not completely susceptible to either concentration of the extract. DMSO 5%, as expected, showed increases in both dead and dying cells, resulting in a significant decrease in living cells.

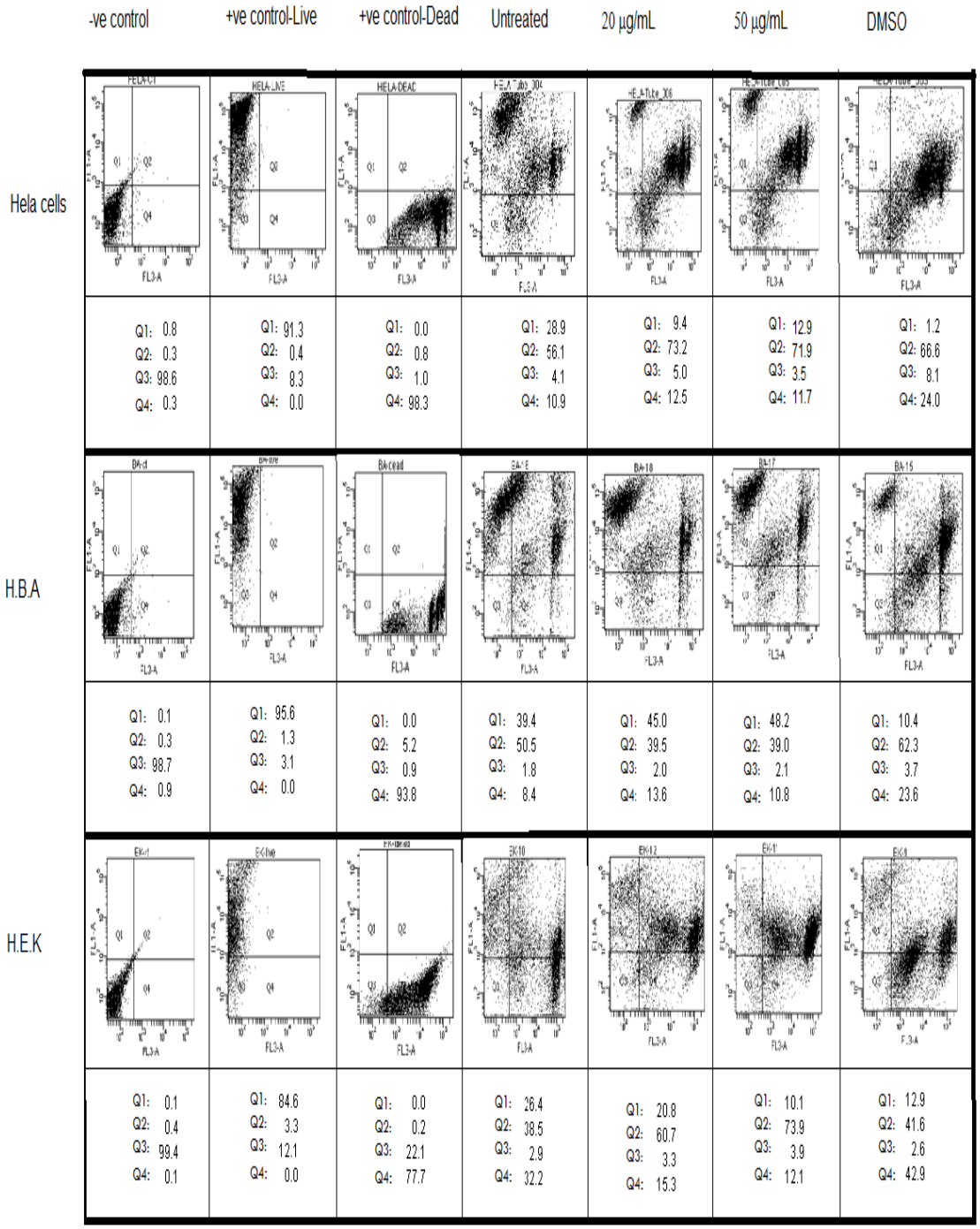



Figure 4: Cytotoxic effect on human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (H.B.A), and human embryonic kidney (H.E.K) cell lines using 20 µg/mL and 50 µg/mL of *Equisetum arvense* methanol extract, and DMSO 5% detected by FACS. Numbers indicate relative percentages in each quadrant (Q1 indicates living cells , Q2 indicates dying cells , Q3 indicates staining errors , Q4 indicates dead cells ).

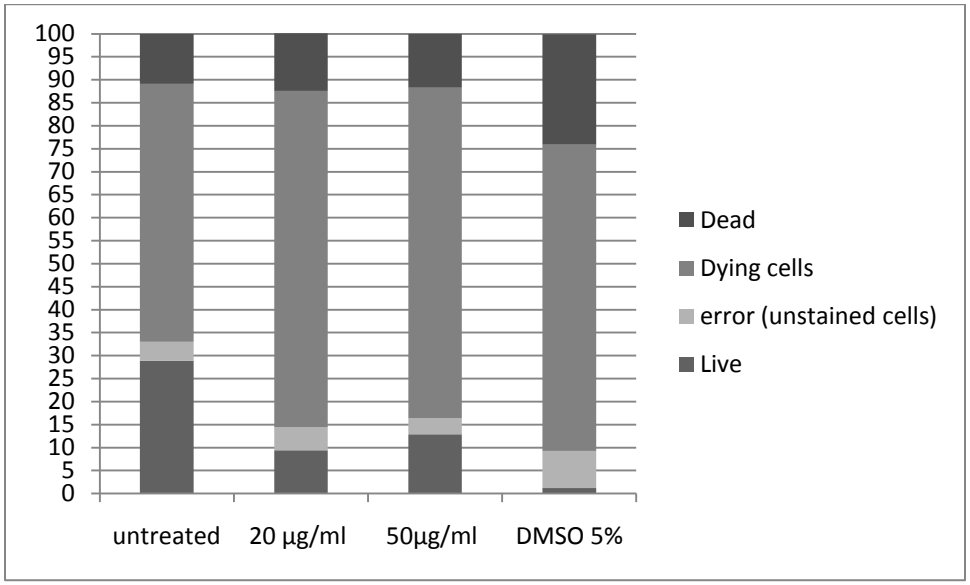



Figure 5: Cytotoxic effect on human cervical adenocarcinoma (HeLa) cancer cell line using 20 µg/mL , 50 µg/mL of *Equisetum arvense* methanol extract and DMSO 5%. Column represents the percentage of living, dead, and dying cells from overall cell count detected by FACS.

Surprisingly, despite the significant cytotoxic effect observed by microscopic examination on the breast cancer cell line, this assay showed no profound decrease in living cells or increase in dead cells compared to untreated cells (Figure 6).

For the human embryonic kidney cell line, we observed a decrease in the percentage of living cells when treated with extract, which was a dose-dependent effect. 5% DMSO showed a stronger cytotoxic effect, manifested by significant increases in the percentage of dead cells; consistent with microscopic examination. The decrease in dead cells noticed might indicate possible improper staining on some wells (Figure 7).

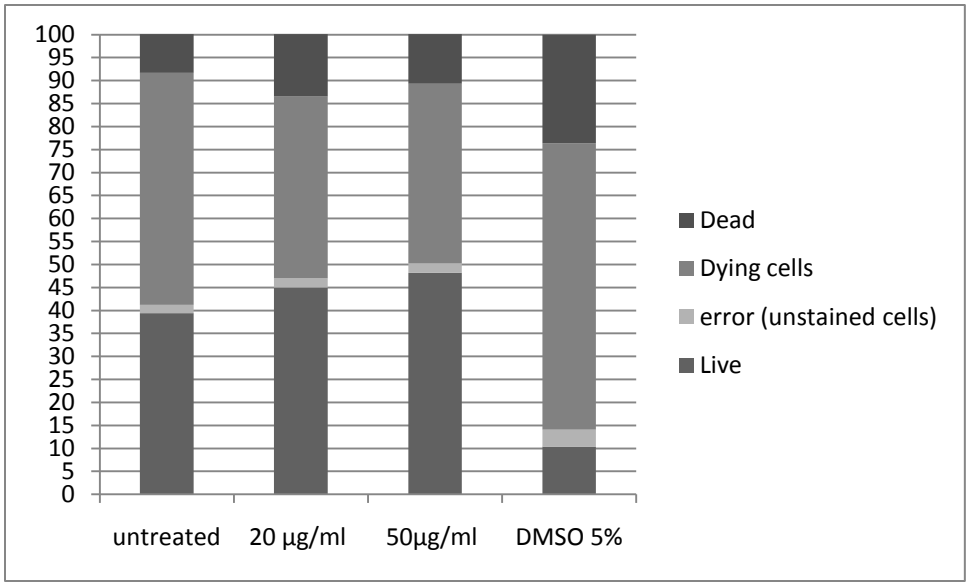



Figure 6: Cytotoxic effect on human breast adenocarcinoma (H.B.A) cancer cell line using 20 µg/mL, 50 µg/mL of *Equisetum arvense* methanol extract and DMSO 5%. Column represents the percentage of living, dead, and dying cells from overall cell count detected by FACS.

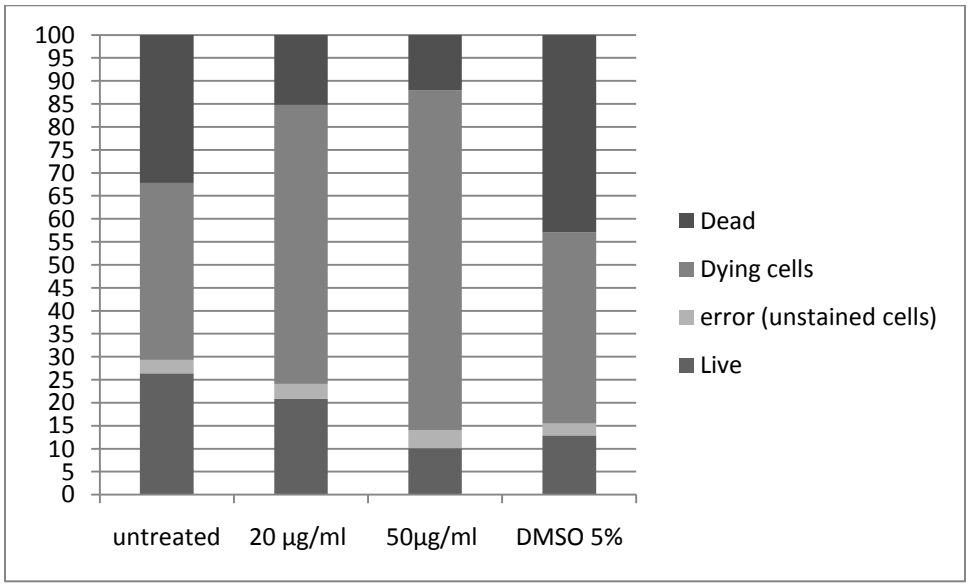



Figure 7: Cytotoxic effect on human embryonic kidney (H.E.K) cell line using 20 µg/mL, and 50 µg/mL of *Equisetum arvense* methanol extract and DMSO 5%. Column represents the percentage of living, dead, and dying cells from overall cell count detected by FACS.

In addition to measuring the cytotoxicity using the Live/Dead cells assay, we also confirmed this data using the Alamar blue assay. Analysis of cell proliferation and cytotoxicity is a vital step in evaluating cellular health and alamar blue is a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. For this round of experiments we exposed four different cell lines (the three cell lines that were tested in the previous round, in addition to lung fibroblast cell line as normal cell model) to 20 µg/mL, 50ug/mL and 100ug/mL of horsetail extract for 3 days. Higher concentration of DMSO (50% instead of 5% )was used this round to increase the effectiveness as a cytotoxic positive control 10% v/v alamar blue in culture media were added to cells after washing twice with PBS. Flourescence was read by xMark™ Microplate Absorbance Spectrophotometer from Bio-Rad at 570 nm and 600 nm with 0.1 path length correction. Flourescence is directly proportional with viability.

As illustrated in Figure 8, all concentrations of the extract tested resulted in more than 40% cytotoxic effect (Figure 8). The effect on HeLa and H.E.K cells was concentration dependent; while H.B.A and H.L.F cell lines did not show any significant difference among different concentrations. The extract concentration of 100 µg/ml did not show any increase in cytotoxicity compared to other concentrations. Instead, an increase in cell viability was noticed in three cell lines. This observation might suggest possible contamination of the prepared extract with a contaminant that may have induced the viability of cells.

Human embryonic kidney was found to be the most sensitive to the extracts used; which is consistent with microscopical examination and Live/Dead assay. This cytotoxic activity observed on H.E.K cell lines might suggest that the plant extract possess nephrotoxic effect. Toxicity toward kidney cells is of great importance specifically for this plant; since it's used traditionally without any medical supervision for the treatment of kidney ailments, which raises the issues of safety and feasibility of the use of this plant. However, although H.E.K originally derived from normal human embryonic kidney cells, it undergo transformation by adenovirus, thus making these cells not a particularly good model for normal cells. Further studies on normal kidney cells (such as renal inner medullary collecting duct (mIMCD3) cells) must be conducted to evaluate the nephrotoxic effect. The significant effect of the plant extract on H.E.K cells might also suggest cytotoxic effect toward kidney cancer cells. Since H.E.K are not a good model for cancer or normal cells, further studies are warranted on kidney cancer cell lines such as the human kidney cancer lines A498, and CAKI-1 in order to analyze anticancer activity toward kidney cancers.

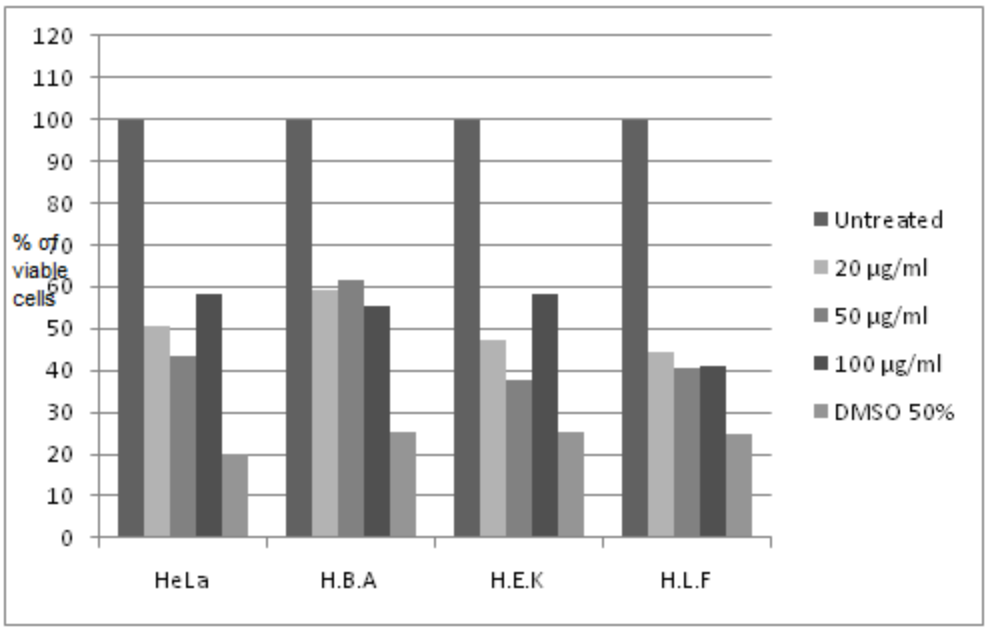


Figure 8: Cytotoxic activity of 20 µg/mL , 50 µg/mL, and 100 µg/mL of *Equisetum arvense* methanol extract, and DMSO 50% on human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (H.B.A), human embryonic kidney (H.E.K), and human lung fibroblast (H.L.F) cell lines. Column represents percent difference in reduction of alamar blue dye between treated and control cells (= % of viable cells). (n=3)

To evaluate the effect at a lower extract concentration, the same experiment was repeated using 10 µg/ml plant extract. At low concentrations, H.E.K was the most susceptible cell line (Figure 9) with 12 % cytotoxicity observed. Since changes were observed in neither H.L.F nor HeLa cell lines, this might indicate a selectivity of this extract against the human embryonic kidney cell line.

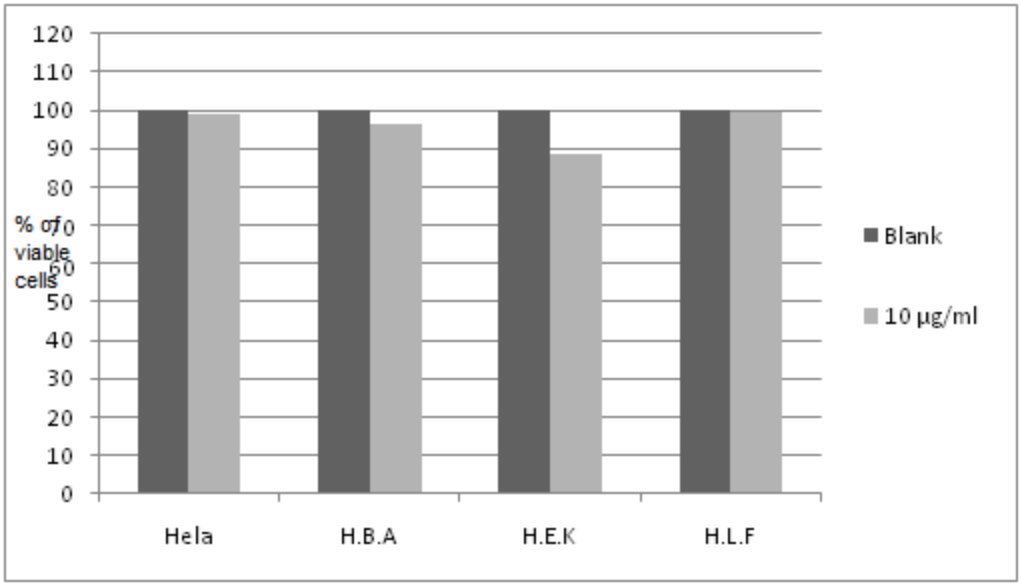



Figure 9: Cytotoxic activity of 10 µg/mL of *Equisetum arvense* methanol extract on human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (H.B.A), human embryonic kidney (H.E.K), and human lung fibroblast (H.L.F) cell lines. Column represents percent difference in reduction of alamar blue dye between treated and control cells (= % of viable cells). (n=6)

### 3.2 Antibacterial activity

This study was conducted to investigate the *in vitro* antibacterial activity of methanolic extract of *Equisetum arvense*. As mentioned earlier, there have been many studies that show antibacterial activity the plant on many strains of gram negative and positive bacteria; however most of them were of the essential oil of the plant-not the methanolic extract. Methanol was used here to extract the plant, and water was used to prepare different concentrations of that extract for the screening. It was tested against the Gram negative bacteria *Escherichia coli*.

The results for antibacterial activity screening of *Equisetum arvense* of the selected concentration is shown in Figure 10. Among the five concentrations used, only the disc

that was dipped in 1g/ml of plant extract in water showed significant antibacterial effect. 1g/ml was the highest concentration used.

This antibacterial activity agrees with previous studies that showed no activity of the plant extract against *E.coli* [17, 25]. The type of extract used was different; Lotfipour et al. 2008 [25] tested chloroform extract, and Canadanovic et al. 2009 [17] used ethyl acetate, n-butanol, petroleum ether, and water extracts. This can suggest that the methanolic extract contains a bioactive compound with a potential antibacterial activity that may not be present in other extracts. This might be explained by a preferable polarity or other molecular property of methanol as a solvent for the active compound. It is worth mentioning also that Canadanovic et al. 2009 used discs impregnated with 10 microliters of 100 mg/ml; which is a lower extract concentration.

The effect of the methanol extract however, is promising and isolation of the active constituent could be the subject of additional research.

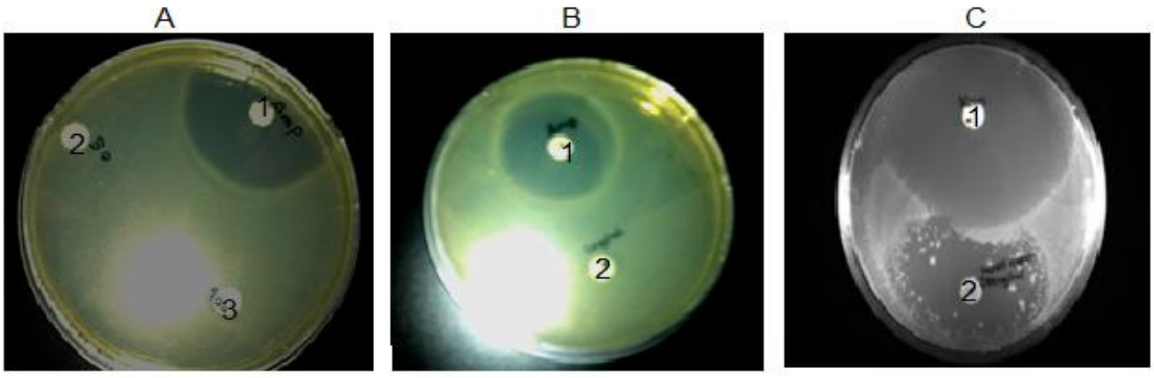



Figure 10: Antibacterial activity of different concentrations of *Equisetum arvense* methanol extract on *Escherechia coli* using disc diffusion assay. A1: Ampicillin 100 µg/ml (control) , A2: 50 µg/ml plant extract , A3: 100 µg/ml plant extract , B1: Ampicillin 100 µg/ml (control) , B2: 500 mg/ml plant extract , C1: Kanamycin 50 µg/ml (control) , C2: 1g/ml plant extract .

# Chapter VI

# CONCLUSIONS

In conclusion, some people in Saudi Arabia use the medicinal plant *Equisetum arvense* for several indications. These preliminary studies aimed to screen a methanolic extract of the plant for activity as antimicrobial and cytotoxic agents on four cell lines. The study shows that *E. arvense* can be a potential novel source of antibacterial and anticancer compounds. It also might suggest nephrotoxic effect possessed by the extract. However more research must be conducted to verify these findings and determine the specific molecules responsible for the bioactivity.

These preliminary studies must be repeated several times to determine significance and reproducibility of the observed activity, and to eliminate any related experimental errors. Other supporting cytotoxicity assays must be used, e.g: measuring lactate dehydrogenase (LDH) release, using MTS or sulforhodamine B (SRB) assays. For antibacterial activity, the next step would be conducting broth dilution test to determine the minimum inhibitory concentration (MIC) of the plant extract against the selected strain. It is also suggested to use various separation processes using HPLC to identify the compounds present in the plant and test them for biological activity, to determine the responsible bioactive molecule.

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