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In Vitro Cytotoxic Activity of Total Flavonoid from *Equisetum Arvense* Extract

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Abstract

Background: Normally happening substances like flavonoids are regarded as active candidates for the treatment and prevention of cancer The purpose of this study was to see how Iraqi *E. arvense* total flavonoid affected cell lines biologically and human lung fibroblast normal cell line (WISH).

Methods: Plant powder was extracted by reflex apparatus, then thin-layer chromatography (TLC) was used to determine total flavonoids. Cytotoxicity assay (MTT) was used to determine the cytotoxic activity of the prepared plant against human breast cancer (MCF-7), cells human cervix cancer (HELA), human colon cancer (Caco-2) and human lung fibroblast normal cell line (WISH).

Results: The flavonoids Rutin, Quercetin, Kaempferol, and luteolin were detected using the Thin Layer Chromatography (TLC) technique. In contrast to the negative control, the extract inhibited cell growth to a highest of 82.158% for MCF-7 and 61.360% for Caco-2 at the concentration (100 µg/ml), and (54.880%) for Hela cell line at the concentration (100 µg/ml). In addition, the concentration (6.25 µg/ml) of total flavonoid extract produced a decrease in the growth of the normal WISH cell line to reach (1.094%).

Conclusions: *Equisetum arvense* contain high amounts of flavonoids, the qualification of some flavonoids compounds was detected using TLC. The total flavonoids showed significant cytotoxic activity against various types of cancer cell lines and normal cell line *in vitro*, the antitumor activity was highly efficient in a dose and cell type dependent manner.

Keywords: *Equisetum arvense* L, Caco2, Hela, Total flavonoid, MCF-7, WISH.

Introduction

Cancer is a category of illnesses defined by uncontrolled development and multiplication of aberrant cells that can cause death (1). Traditional methods for treating cancer include chemotherapy, radiation, surgery, immunotherapy, and hormone therapy. Operating surgery is unable to get rid of the unseen nests of tumors. On the other hand, chemotherapy treatments destroy the tumor and normal cells equally, and tumor cells can acquire resistance to these medications (2). Natural materials such as plants include a variety of phytochemicals that have lately been investigated as effective free radical scavengers, antioxidants, addition to immunomodulating agents, as well as being tested for their radioprotective benefits. Herbal medications are therefore a viable alternative to synthetic substances, as they are either harmless or less harmful than their synthetic equivalents (3). Equisetum is the only living genus belongs the family Equisetaceae, order

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Equisetales, class Equisetopsida. *E. arvense* is a medicinal plant that was found in America, Northern Africa, and Asia (4). This plant commonly named horsetail and scouring rush, is traditionally uses for a wide range of illnesses treatment as a diuretic, and edematous, anti-inflammatory, and demineralizing. *E. arvense* is extensively utilized in modern medicine as a medication for liver illnesses, gastrointestinal disorders, as well as tumor prevention and therapy due to its significant pharmacological benefits (5). Thus, phytosterols, triterpenoids, alkaloids, tannin, and phenolics are found in *E. arvense*, while styrylpyrones and flavonoids are types of phenolics (6, 7). Flavonoids are a group of chemicals found in abundance in nature. Many studies have been done to investigate flavonoids' biological activity as an anti-diabetic, cardio-protective, antiviral, and anti-tumor. Various research have been done to determine the daily consumption ranges of flavonoids which get from different types of foods such as tomatoes, apples, and onions hence it was demonstrated that the range of daily consumption is between 20 to 500 mg (8, 9).

The goal of this work was to identify and quantify total flavonoids isolated from Iraqi-grown plants *E. arvense* (fern) and estimate they're anticancer on four different cell lines.

Materials and Methods

Collecting of plants

The studied samples were brought from Hagi Omeran town in Kurdistan region North of Iraq in the summer and identified by Dr. Hoshyar Abdullah Azeez, the Faculty of Pharmacy/ Sulaimaniyah University, Iraq. Arial parts were dried at room temperature (25 °C) and then was crushed using a mortar and pistil. Then they're placed in sterile vessels and kept out of direct sunlight until they're ready to use.

Extraction of flavonoids from E. arvense

Powdered (aerial part 100 g) was extracted by reflex apparatus with aqueous hydrochloric acid (10%) till exhaustion for 3 days. Then filter and extracted with ethyl acetate (30 mL × 3), the upper layer (ethyl acetate layer) was separated by a separatory funnel then wash by water, it was dried by using anhydrous sodium sulphate and labeled as Ethyl acetate extract then evaporated by a rotary evaporator to yield a sticky mass (10).

TLC technique to identify total flavonoids.

In this experiment, thin-layer chromatography (TLC) was used to determine total flavonoids. It was prepared in stock solution by dissolving 0.15 g of extracted total flavonoids in a 5 ml mixture of ethanol and water 1:1. However, 70% ethanol was used to prepare the standard solutions such as kaempferol, rutin, luteolin, and quercetin. Aluminum TLC plates (20 x 20 cm with thickness of 0.1 mm) and fluorescent silica gel TLC were used to identify the flavonoids. A mixture of chloroform: glacial acetic acid: Formic acid (88: 7: 5) was utilized as a mobile phase. The flavonoids kind were determined by calculating the Retention Factor (RF) of the spots on the TLC plates and comparing it with the standards RF which have been determined in the literature. Retention Factor (RF) value is calculated by dividing the distance travelled by each flavonoid in each model phase to the distance traveled by the solvent:

$$R\ value = \frac{\text{Distance taveled by a substace}}{\text{Distance traveled by the eluent}}$$

Each compound can be identified by exposing the silica sheet to UV light at a wavelength of 254 nm as a coloured spot. In comparison to a standard, the outcome appears as clear spots under UV light (11).

Cytotoxicity assay (MTT)

Cytotoxicity assay (MTT) was used to determine the cytotoxic activity of the prepared stock solution. RPMI-1640 medium completed with 10% fetal bovine serum and 10 µg/ml of Ciprofloxacin was used to culture all four cell lines, human breast cancer (MCF-7) cells, human lung fibroblast normal cell line (WISH), human colon cancer cells, and human cervix cancer (HELA) cells. At 37 °C, cells were incubated in a 5% CO_2 environment with 100% humidity. They were seeded twice a week and cultured in flat 96-well micro-titer plates (10^4-10^6 cells/well) in

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a total volume of 200 µL of complete culture media, with MTT used to measure cell number and viability. Plates were incubated 24 hours, at 37 °C with 5% CO_2.

The media was withdrawn, and the wells were rinsed once with PBS before adding two-fold serial concentrations of the materials under test (0, 6.5, 12.5, 25, 50, and 100 µg/ml) to the monolayer cell. Each dose was tested in triplicate and incubated at 37 ºC in, a 5% CO_2 incubator for 24 hours. After that, MTT solution (20 µL) was added to each well and mixed well, then put in the incubator again in the same conditions for 4 hours more. Next step, acidic isopropanol (50 µL) was added to dissolve the formed crystals then incubated in the dark at 37 ºC for 4 hours. Finally, the absorbance at 570 nm (background wavelength was 630 nm) was read using spectrophotometer. Sigmoidal concentration-response curve fitting models was used to calculate the IC50 values (Sigmaplot software) (12). Thus, it was applied the below equation to calculate the growth ratio (13):

$$\text{Growth inhibition Rate\%} = \frac{\text{control} - \text{test cell}}{\text{control}} \times 100$$

Results

Total flavonoids from TLC

In comparison to standard flavonoids, total pure flavonoids were extracted from *E. arvense* and qualitatively detected by TLC, indicating that the plant was rich in flavonoids, particularly Kaempferol (K), luteolin (L), Rutin (R), and Quercetin (Q). Table 1 shows the Rf ratios for extracted and standard flavonoids.

Table 1. R_f values for standard and extracted flavonoid from *E. arvense*.

Spot Number	T	Standard Rutin	Standard Quercetin	Standard Kaempferol	Standard Luteolin
1	0.24	**0.32**		**0.74**	**0.66**
2	0.3		**0.48**		
3	0.56				
4	0.66		**0.56**		
5	0.78				
6	0.8		**0.7**		

Cytotoxic effect

The half-maximal inhibitory concentration (IC50) of total flavonoid expression elucidates the concentration of substances that inhibit 50 percent of cell proliferation relative to untreated cells (14). Table 1 shows statistical data and IC50 values for total flavonoid and their concentrations in the inhibition of cancer cell lines,

Table 2. Influence of different concentrations of total flavonoid extract in the viability of Caco2, Hela, MCF-7 and WISH cells line after 24 hrs of exposure time.

Concentration (µg/ml)	Mean±SE of Viability (%) for Caco2	Mean±SE of Viability (%) for Hela	Mean±SE of Viability (%) for MCF-7	Mean±SE of Viability (%) for WISH
0	100±0.00 a	100±0.00 a	100±0.00 a	100±0.00 a
6.25	78.97±3.49 b	96.74±4.12 ab	67.62±2.59 b	98.90±2.96 a
12.5	65.24±3.08 c	92.62±3.58 ab	61.01±3.76 b	95.62±3.18 a
25	56.86±2.37 d	90.02±3.09 b	42.29±2.85 c	93.02±3.66 a
50	49.57±2.94 d	73.96±4.13 c	36.56±1.97 c	59.50±2.57 b
100	38.63±1.89 d	45.11±2.61 d	17.84±0.89 d	18.05±1.07 c
LSD value	8.924 **	8.593 **	9.422 **	9.672 **
P-value	0.0001	0.0001	0.0001	0.0001

** ($p \leq 0.01$), This means having different letters in the same column differed significantly.

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The interesting result is that in the growing Caco2 cell line, the extracted flavonoids make obvious inhibition in all concertation that were used. The best results were got at 100 µg/ml concentration when the maximum inhibition was reached (61.36088%). However, the MCF-7 cell line reached maximum inhibition (82.15859%) at the above concentration. On the other hand, the Hela cell line was demonstrated (54.8807%) maximum growth inhibition at a concentration of 100 µg/ml. Thus 6.25 µg/ ml concentration shows the lowest growth inhibition percentage which is (3.2538 %).

Morphological Changes

After exposure to *E. arvense* total flavonoids for 24 hr, many cells featured cytoplasmic shrinkage and either detached from each other or hovered in the medium. With a rich dose, there was a considerable surge in the percentage of rounded cells with gradual nuclear shrinkage and clear degradation in cell number as shown in Figure 1.

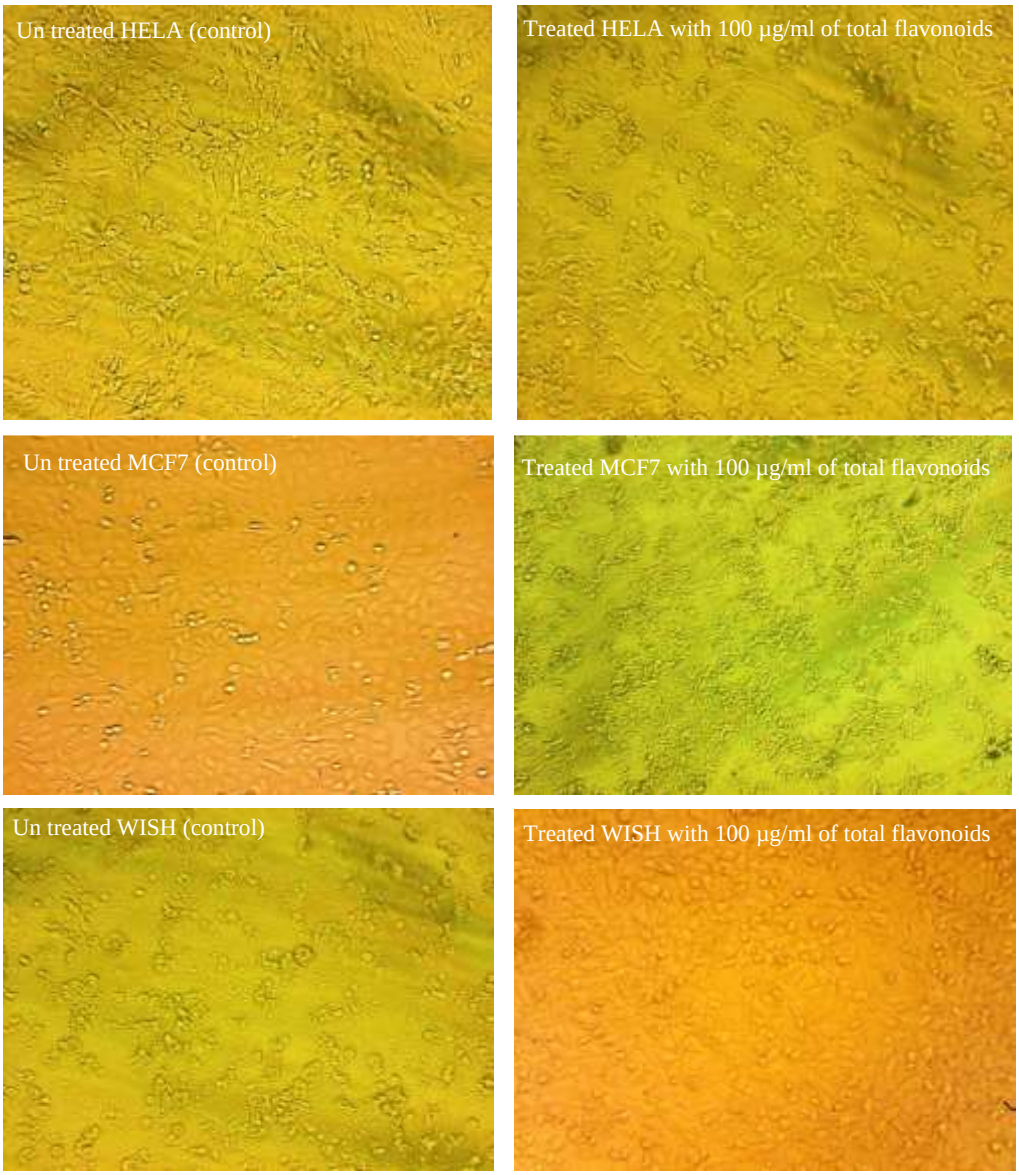

Fig. 1. Inverted microscopy image of HELA, MCF-7 and WISH treated and untreated (control) cell lines respectively. Morphology was visualized and photographed under an inverted microscope (magnification, x100).

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Discussion

The Hormetic effect is a phenomenon that was noticed in the current investigation and contributed to the cell's activity (15). Growth was stimulated at low doses whereas growth was inhibited at higher concentrations in this sort of action. It was a biological effect in toxicology that represented a contrasting impact between growth stimulation (30-60%) and control cell growth. The greater doses, on the other hand, exhibited a partial or full suppression of growth (16). Polyphenolic chemicals found in plants, such as flavonoids, help to prevent cancer at all stages (initiation, promotion, and progression). By raising intracellular ROS levels, polyphenols, especially flavonoids, can induce cytotoxicity. Others have proposed that flavonoids, such as quercetin, trigger S phase arrest in cancer cells during cell cycle development. Others discovered that interacts directly with DNA and maybe one of the ways of causing apoptosis. This suggests that flavonoids have cancer-blocking and -suppressing properties, prompting researchers to focus their efforts on them.

To conclude, total flavonoids were successfully extracted from *E. arvense* plant using a mixture of organic solvents. The purpose of this study was to see how Iraqi *E. arvense* total flavonoid affected cell lines biologically and human lung fibroblast normal cell line (WISH)). There were instances when toxicity appeared to be dosage and type dependent, which might be attributable to cell type and sensitivity to the extract. cytotoxically (human breast cancer (MCF-7), human cervix cancer (HELA), human colon cancer (Caco-2).

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