

ANTICANCER, ANTIBACTERIAL, AND ANTIFUNGAL ACTIVITIES OF ARUM PALAESTINUM PLANT EXTRACTS

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ABSTRACT

Traditional natural remedies have long played an important role in the treatment of cancer and infectious diseases. A part from the high cost and undesirable side effects associated with synthetic drugs, increased interest has intensified to determine the biological effects of plant extracts on malignant cells as alternative for conventional drugs used in the markets. The medicinal properties of Arum Palaestinum Boiss were in-vitro investigated in this research project. Arum palaestinum is chosen based on its use in traditional palestinian herbal medicine. The leaves of this plant were air dried in the shade and then three types of extract were obtained and their antimicrobial and for anticancer activity testing. Investigations on three different cancer cell lines (C2C12, 3T3-L1, Hela) revealed direct inhibitory effect of the extracts.

This effect differs according to concentrations used. Aqueous boiled extract was more effective at lower extract concentrations as compared to other two extracts. Arum palaestinum plant extracts showed no inhibitory effect on bacterial species (Escherichia coli,

Staphylococcus aureus and Pseudomonas aeruginosa) as well as yeasts (Candida albicans). In conclusion, *Arum palaestinum* has potentially anticancer effect. Further investigations are required to confirm this conclusion and to elucidate the mechanisms of actions and toxicity of this herb.

KEYWORDS: *Arum Palaestinum*, anticancer, herbal medicine, crude extract.

INTRODUCTION

Many plants and medicinal herbal extracts are used in the treatment of various ailments. Herbal medicine (botanical medicine or phytomedicine) is a concept used to describe the usage of plant's roots, seeds, bark, stems, flowers, or leaves for medicinal purposes. Quality control and analytical methods reinforced the significance of applying herbal medicine in clinical research. Recent advances as well as proper and careful use of herbs has contributed to the success in the treatment of various diseases.^{[1][2]}

Herbal remedies can be utilized to manage many health problems such as facilitating digestion, decreasing pain sensation, stimulants, increasing libido, cleansing the bowel and many other purposes.^[3] In addition, natural remedies are preferred over synthetic drugs, which can be harmful or cause undesirable side effects. *Arum palaestinum* as shown in Figure 1, is one of these medicinal plants that have been frequently used in traditional medicine.^{[3][4]} It is widely used in traditional Arabic Palestinian herbal medicine for the treatment of diverse disease conditions such as stomach acidity, atherosclerosis, cancer, diabetes and food toxicity.^[5]

Arum palaestinum Boiss (family: Araceae, Figure 1), is a low-growing tuberous perennial plants, 20-60 cm tall with heart or arrow shaped leaves having bitter and burning taste. It has an erect, slender spadix surrounded by a spathe, which is pale green with purplish spots. *Arum* genus has 26 different species that are generally recognized and it is native to southern Europe, northern Africa and has very high species diversity in the Mediterranean region.^[3,4]



Fig. 1: *Arum palaestinum* Plant, March 2016.

Many studies have investigated *Arum palaestinum* inhibitory effect on certain types of cancer cells and various types of fungal and bacterial isolates.^{[5]–[8]} The phytochemical screening of Arum plant revealed that these plants contain polyphenols, glycosides (flavonoids, saponin and cyanogenic groups), isoprenoids or terpenoids. Isoprenoids consist primarily from isoprene units which exhibit antimicrobial, antiviral and antiprotozoal activities in certain plant species.^[9]

The aims of this research project were to prepare three types of crude extracts from this plant and investigate in-vitro its anticancer, antibacterial or antifungal effects.

Furthermore, the anticancer activities of *Arum palaestinum* plant extracts were evaluated against three types of cancer cell lines; The first cell line is C2C1-2 mouse myoblasts (ATCC® CRL-1772™).^[10]

The second type of cancer cell lines to be used for investigation is Hela cell line (ATCC® CRMCL2™), a cervix tissue taken from a black human female, 31 years old, revealing an epithelial cell type morphology which is related to adenocarcinoma disease.^[9,10] Cervical cancer is the third most common cancer that affects women worldwide (frequency differs between developed and non-developed Treatment for cervical cancer depends on cancer growth, but in general it would be treated by surgery (hysterectomy and pelvic lymph nodes removal), chemotherapy and radiation therapy.^{[11],[12]}

The third type of cell lines is 3T3-L1 cell line (ATCC® CL-173™). It is an embryo tissue taken from *Mus musculus*, mouse, revealing a fibroblast cell type morphology which is a

avored model for metabolism and obesity research since they can be chemically induced to differentiate into adipocytes.^[13]

MATERIALS AND METHODS

Microorganisms: *Escherichia coli* ATCC 25789, *Pseudomonas aeruginosa*, ATCC 9027, *Staphylococcus aureus* ATCC 29737 and, *Candida albicans* ATCC 10231.

Cancer cell lines: C2C12 cell line (ATCC® CRL-1772™), Hela cell line (ATCC® CRM CCL2™) and 3T3-L1 (ATCC® CL-173™).

Chemicals: Methanol, Dichloromethane, ethanol, gentamicin, ampicillin G, amphotericin B, trypan blue, 0.25% trypsin-EDTA solution.

Plant Extract Preparation

Leaves of *Arum Palaestinum* were collected from Salfect region in the West Bank, Palestine during the months of February and March 2015. The plant leaves were air-dried in the shade for three weeks for appropriate phytochemical extraction. The dried leaves were then pulverized to powder using a grinder. The resulting powder was stored at room temperature in a well-sealed plastic container until use.

Extracts Preparation

Aqueous (boiled and cold) as well as organic (Methanol: Dichloromethane, 1:1 v/v) extracts were prepared and tested for their activity as antimicrobial and anti-cancer agent.

Organic Extract (Methanol: Dichloromethane, 1:1 v/v)

An amount of 20g of the powdered leaves were added to 200 ml of Methanol/Dichloromethane mixture and left at room temperature for 24 hours. The mixture was then filtered by suction using Buchner filter cone, and filtering paper (Whatman®, 125 mm). The accumulated paste was then soaked in absolute Methanol for 10-20 minutes at room temperature and filtered again as above. The two filtrates were then combined and placed in the rotary evaporator (RE300/MS) to dry under vacuum and temperature of 60°C and then stored at -20°C until use.

Aqueous – boiled sterile distilled water

An amount of 20g of the powdered leaves were added to 200 ml boiled water and left at room temperature for 15 minutes. The mixture was then filtered twice by suction using Buchner

filter cone and filtering paper (Whatman®, 125 mm). The filtrate was then placed in the rotary evaporator (RE300/MS) to dry under vacuum and temperature of 60°C and then stored at -20°C until use.

Aqueous – cold sterile distilled water

An amount of 20g of the powdered leaves were added to 200 ml of sterile distilled water and left at room temperature for 5 days. The mixture was then filtered twice by suction using Buchner filter cone and filtering paper (Whatman®, 125 mm). The filtrate was then dried on watch glass for 48 hours 25°C and then stored at -20°C until use.

Biological activity

Testing organic extract of *Arum Palaestinum* on bacterial and fungal isolates

Two species of Gram negative bacteria (*E. coli*, ATCC 25789 and *P. aeruginosa*, ATCC 9027), one Gram positive bacterial species (*S. aureus*, ATCC 29737) and one yeast species (*C. albicans*, ATCC 10231) were plated on appropriate media for 24 hours.

The extract was prepared by weighting 0.32 g of organic extract and dissolved in enough amount of DMSO (~2µL). Sterile distilled water was added to make a stock of 2000µg/ml. This preparation was filtered by Minisart, non-pyrogenic 0.45 µm filters, for sterilization. The stock was serially diluted to make working concentrations of: 2000 µg/ml, 1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml and 31.25µg/ml.

Bacterial and candida suspensions equivalent to 0.5 McFarland standard were prepared in sterile distilled water and swabbed immediately on the surface of MH agar. The plates were left for 10-15 minutes to absorb the suspensions. A Whatman, 125 mm filter paper was cut into discs of 6 mm and sterilized in the autoclave. The discs were placed on the surface of the MH plates, and 6µl of each concentration was added to the discs. After few minutes, the plates were incubated in an inverted position at 37° C for 24 hours in non-CO₂ incubator. The zone of inhibition was then measured in millimeters using Vernier Caliper.

Testing organic extract of *Arum Palaestinum* on bacterial and fungal isolates (higher concentrations)

The same procedure mentioned above was followed but with higher concentrations. The working concentrations were: 10000µg/ml, 8000µg/ml, 6000µg/ml, 4000µg/ml and 2000µg/ml.

Testing of aqueous- cold sterile distilled water extract of Arum Palaestinum on bacterial and fungal isolates.

Two species of Gram negative bacteria (*E. coli*, ATCC 25789 and *P.aeruginosa*, ATCC 9027), one Gram positive bacterial species (*S. aureus*, ATCC 29737) and one yeast species (*C. albicans*, ATCC 10231) were plated on appropriate media for 24hours.

The extract was prepared by weighing 100mg of aqueous- cold sterile distilled water extract and dissolved in enough amount of sterile distilled water (~1mL). The crude extract is ready with concentration of 100µg/µl. This preparation was filtered by Minisart filter. The same procedure was followed regarding bacterial and fungal suspension preparation. A 10 µL and 5µL were applied on 6 mm formed filter discs in order to obtain concentrations of 1000µg/ml and 500µg/ml. A stock of 50µg/ml was also prepared, a 5µL and 2.5µL were applied in order to obtain concentrations of 250µg/ml and 125µg/ml, respectively. In addition, a stock of 16µg/ml was prepared. A 4µL, 2µL and 1µL were applied from the later in order to obtain concentrations of 64µg/ml, 32µg/ml and 16µg/ml, respectively on each disk of four different species.

Testing aqueous-cold sterile distilled water extract of Arum Palaestinum on bacterial and fungal isolates (higher concentrations)

The same procedure mentioned above was followed but with higher concentrations. The working concentrations were: 10000µg/ml, 8000µg/ml, 6000µg/ml, 4000µg/ml and 2000µg/ml.

Testing of aqueous- boiled water extract on Arum palaestinum on bacterial and isolates.

The Same procedure mentioned above was followed, with same concentrations of aqueous-cold sterile distilled water extracts.

Tissue culture

Cells in culture require enriched culture media that can support the growth of the cells to be tested. These types of media usually contain fetal bovine serum that contains nutrients, vitamins, proteins, lipids, amino acids, minerals, growth factors that are needed for cell growth.

RPMI 1640 (Sigma, contains L-glutamine and sodium bicarbonate), supplemented with 5% heat inactivated fetal bovine serum (FBS) and antibiotic antimycotic mixture (Sigma, 100X,

1ml/100 ml medium) was prepared. Subsequent to cell growth and monolayer formation, Trypsin was applied to the cell culture flasks for 5 to 10 minutes to detach cells, then neutralized with equal amounts of complete RPMI medium. Viability was determined by making the proper dilution of the cells (1:5) in Hank's balanced salt solution and trypan blue stain. The mixture was mixed gently and left at room temperature for 5-10 minutes, followed by counting the 4 corner squares in a hemocytometer. The number of cells/ml was determined using the following formula:

$$\text{Cells/ml} = \text{dilution factor} \times \text{average number of cells/square} \times 10^4$$

Cell line inoculation/ general procedure

Cells were removed from liquid nitrogen, rapidly thawed and cultured in 25 mm flask containing RPMI 1640 medium supplemented as mentioned earlier and incubated in a humid incubator at 37° C and 5% CO₂. The cells were then examined under the inverted microscope to check for adherence to the bottom of the flask and to ensure the absence of contamination. At about 70-80% confluency, the cells were collected by adding Trypsin for 5 to 10 minutes in the CO₂ incubator and placed in a sterile conical centrifuge tube. Equal amount of RPMI containing FBS was added to stop the action of the trypsin (FBS contains trypsin inhibitor), and centrifuged at 1200 rpms for 7 minutes. The supernatant was then discarded; the cell sediment was re-suspended in RPMI1640 followed by determination of the cell count and percentage of viability using the hemocytometer. A total number of 1.0×10^5 cells per well was placed in a 12 well cell culture plate and incubated under the proper conditions for 24 to 48 hours or until 70% confluence was achieved. After that, the medium was discarded, the cells were washed with Hanks Balanced Salt Solution (HBSS) and RPMI 1640 containing the various concentrations of the extracts was added. Following 24 hours incubation, the cells were collected according to previously mentioned procedure and counted to determine the total and viable counts.

RESULTS AND DISCUSSION

In this study, the crude extract from the air-dried leaves of *Arum palaestinum* were tested for its antibacterial, antifungal as well as anti-cancer effects on three cancer cell lines (C2C12, 3T3-L1 and Hela).

***Arum palaestinum* extract preparation**

Leaves of *Arum palaestinum* were dried up in the shade and subsequently crude extracts were obtained by various methods as described earlier. Although the same quantity of powdered

leaves was subjected to extraction, the highest yield was obtained by the aqueous boiled method as shown Figure 2. This could be explained mainly by the solubility of substances to be extracted in certain types of solvents (as solubility increases, the collected amount increase). Polarity may play an important role as well. Another possible contributing factor is that during aqueous - boiled extraction, the plant material was soaked in boiled water.

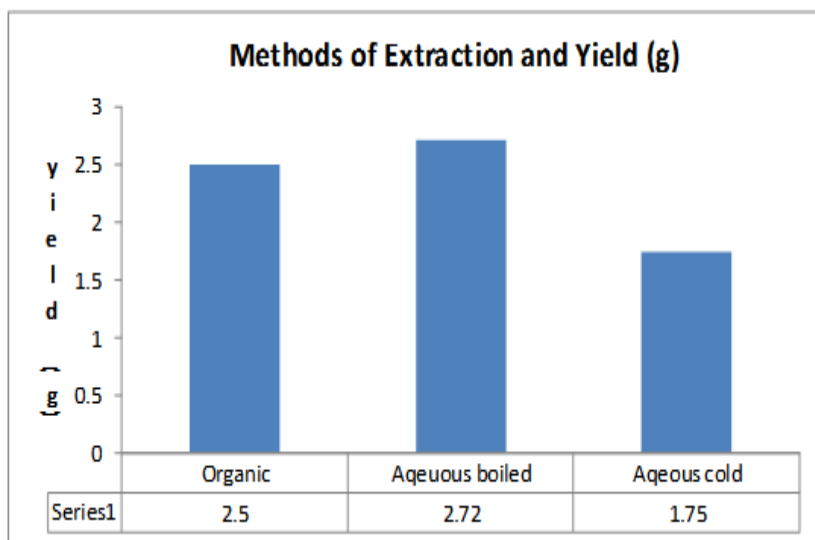


Fig. 2: Total amount (g) of crude extracts of *Arum palaestinum* obtained by three different extraction methods.

Antibacterial and antifungal activities of *Arum palaestinum* extracts

The inhibitory effect of the *Arum Palaestinum* extracts on Gram negative and Gram positive bacterial species (*E. coli*, *S. aureus* and *P. aeruginosa*) were evaluated. In addition, inhibitory effects of these extracts on the fungal isolate (*Candida albicans*) were also investigated. Unfortunately, the plant extracts showed no antibacterial or antifungal activities. Having no antimicrobial effect could be due to the absence of the active chemical component which may exert the expected desired pharmacological effect on the bacterial and fungal isolates, or due to low solubility of the active ingredient in the selected solvents. Our results are compatible to those published by Husein et al. (2014) as they reported that *Arum palaestinum* extract have no obvious inhibitory effects.^[6] Another study conducted in Palestine on 2010 did not reveal any antimicrobial effect of this plant species.^[3] However, a study conducted in Palestine in 2014, took advantage of advanced techniques such as HPLC and GC-MS to isolate the phthalate compounds such as di-isobutyl phthalate and di-n-octyl phthalate from *Arum palaestinum* plant with the highest concentrations were found in the leaves.^[14] These compounds have been reported to have antimicrobial activity. To provide some explanation

for this controversy; it is possible that Araceae family of *Arum palaestinum* represents secondary plant metabolites that include polyphenols and alkaloids. It is noteworthy that some plants which belong to the same family may show different effects due to having certain ingredients in one member of the family but not in the other.

Anticancer activities of *Arum Palaestinum* extracts

Cancer is a devastating health problem worldwide. It is considered as the second leading cause of death after heart diseases globally. Chemotherapeutic agents with or without radiation therapy or surgery are in-use today as the sole anticancer treatment. However, chemotherapy has notable problems such as having poor selectivity that kills both normal and malignant cells. In addition, long term treatment may cause drug-resistance. So, there is a trend among many people toward complementary and alternative medicine and all of this in turn rationalizes the search for potential anticancer agents from natural sources. Many studies have confirmed the presence of anticancer activity of herbs such as *Arum Palaestinum*.^[15]

C2C12 cancer cell lines

As shown in Table 1 and Figure 3, the cell count and percentage of cell inhibition of C2C12 cell line after application of the three different extracts of *Arum Palaestinum*. It is clear that there is significant decrease in the cancer cell lines counts using the three different extracts compared to negative control. This activity is higher at higher concentration compared to lower ones.

It was evident that CaCL2 cell line was not significantly inhibited by the aqueous cold extract as compared to the organic and aqueous boiled extracts. However, the inhibitory effect of the organic extract at 512 ug/ml was higher (82%) than the aqueous boiled extract (65%). At concentration of 256 ug/ml and lower, the inhibitory effects of both extracts were similar.

Table 1: Cell count and % inhibition of C2C12 cell line after the application of the three different extract of *Arum palaestinum*.

Concentration (µg/ml) Cell Count	% inhibition (Organic extract)	Cell/ml (organic extract)	% inhibition (Aqueous boiled extract)	Cell/ ml (Aqueous boiled extract)	% inhibition (Aqueous cold extract)	Cell/ml (Aqueous cold extract)
512	82	12.5x 10 ⁴	65	16.25 x 10 ⁴	29	68.75 x 10 ⁴
256	61	27.5 x 10 ⁴	68	15 x 10 ⁴	20	76.25 x 10 ⁴
128	53	33.75 x 10 ⁴	65	16.25 x 10 ⁴	8	88.75 x 10 ⁴
64	49	36.25 x 10 ⁴	35	30 x 10 ⁴	4	92.5 x 10 ⁴
32	19	57.5 x 10 ⁴	11	41.25 x 10 ⁴	10	86.25 x 10 ⁴
control (0)	0	71.25 x 10 ⁴	0	46.25 x 10 ⁴	0	96.25x10 ⁴

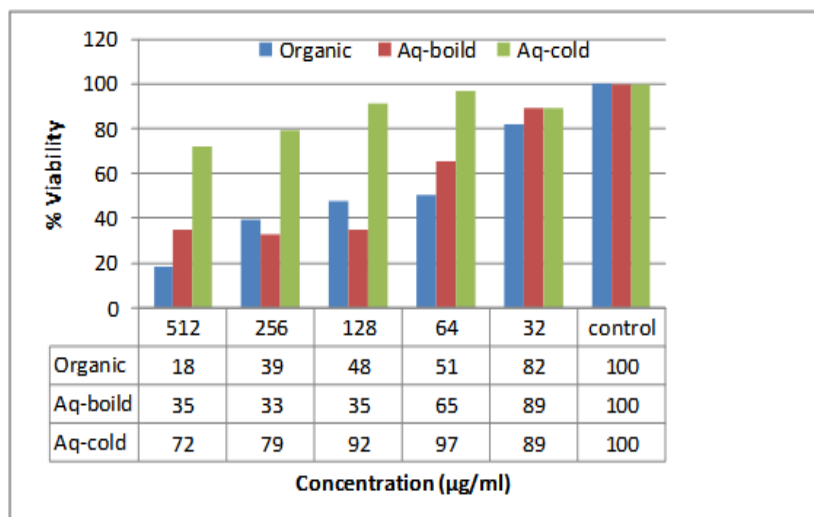


Figure 3: % Cell Viability of C2C12 cell line after the application of the three different extracts of *Arum palaestinum*.

3T3L-1 cell line

Results showed that there is significant decrease in the cancer cell lines count using the three different extracts compared to negative control. This activity increases by increasing the concentration of the extracts. The inhibitory effects of the three extracts on 3T3L-1 cell line is shown in Table 2 and Figure 4.

It was obvious that the pattern of inhibition of this cell line was different than the previous one. The aqueous boiled extracted was far more influential in inhibiting the growth of this cell line at all concentrations used as compared to the other two extracts. Furthermore, the inhibitory effect of the aqueous cold extract on this cell line was noticeable at concentration range of 128 to 512 ug/ml. As for the organic extract, the inhibitory effects were seen only at the highest concentration of 512 ug/ml while the remaining concentrations had lower inhibitory effects than the others.

Table 2: Cell count and % inhibition of 3T3L-1 cell line after the application of the three different extract of *Arum palaestinum*.

Concentration (µg/ml) / Cell count	% inhibition (Organic extract)	Cell/ml (organic extract)	% inhibition (Aqueous boiled)	Cell/ml (Aqueous boiled extract)	% inhibition (Aqueous cold)	Cell/ml (Aqueous cold extract)
512	69	6.25×10^4	71	1.2×10^4	61	1.5×10^4
256	37	12.25×10^4	71	1.2×10^4	64	1.4×10^4
128	25	15×10^4	74	1.0×10^4	45	2.3×10^4
64	25	15×10^4	64	1.4×10^4	19	3.2×10^4
32	12	1.8×10^4	58	1.7×10^4	19	3.2×10^4
control (0)	0	2.0×10^5	0	3.9×10^4	0	3.9×10^4

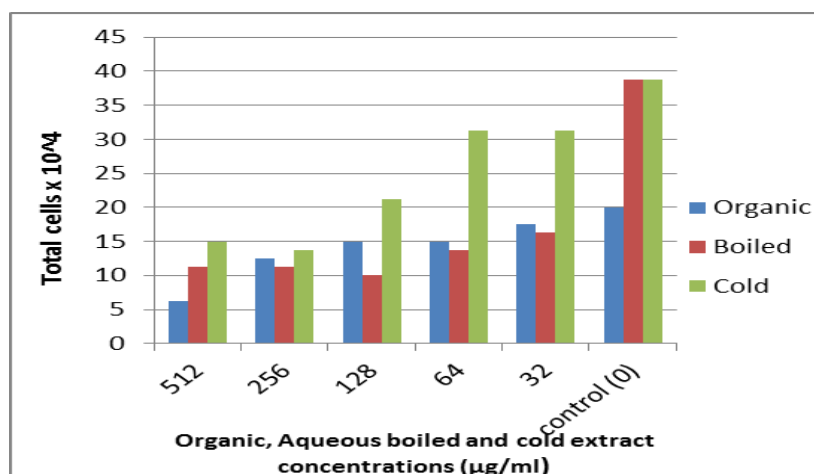


Figure 4: Cell count of 3T3L1 cell line after the application of *Arum palaestinum* three different extracts.

Hela cell line

The results of the inhibitory effects of the extracts used on this cell line is shown in Table 3 and Figure 5. Hela cell line was significantly inhibited by the organic extract at higher concentrations of 512 and 256 µg/ml. At lower concentration its effect was similar to that exerted by the aqueous boiled extract. However, the aqueous cold extract has inhibitory effect on the cells of 62% by its highest concentration of 512 µg/ml whereas the rest of the concentrations tested were negligible.

It is interesting to compare the activity of the three extracts on the studied cell lines using the three method of extraction (organic, cold aqueous, and boiled aqueous). It was found that hot aqueous extract is more effective at lower and higher extract concentration as compared to the aqueous cold extract. An interesting and similar study that has been conducted in Egypt reported that the hot aqueous extract exerted higher anti-cancer activity more than the organic extract for the arum species.^[16]

Table 3: Cell count of Hela cell line after the application of the three different extract of *Arum palaestinum*.

Concentration (µg/ml) Cell count	% inhibition (Organic Extract)	Cell/ml (organic extract)	% inhibition (Aqueous boiled)	Cell/ml (Aqueous boiled extract)	% inhibition (Aqueous cold)	Cell/ml (Aqueous cold extract)
512	81	3.8 x 10 ⁴	53	1.0 x 10 ⁴	62	x 10 ⁴
256	75	5.0 x 10 ⁴	53	1.0 x 10 ⁴	27	2.4 x 10 ⁴
128	56	8.8 x 10 ⁴	47	1.2 x 10 ⁴	15	2.5 x 10 ⁴
64	37	1.3 x 10 ⁴	30	1.5 x 10 ⁴	8	3.0 x 10 ⁴
32	31	1.4 x 10 ⁴	23	1.7 x 10 ⁴	0	3.3 x 10 ⁴
control (0)	0	2.0 x 10 ⁵	0	2.2 x 10 ⁴	0	3.3 x 10 ⁴

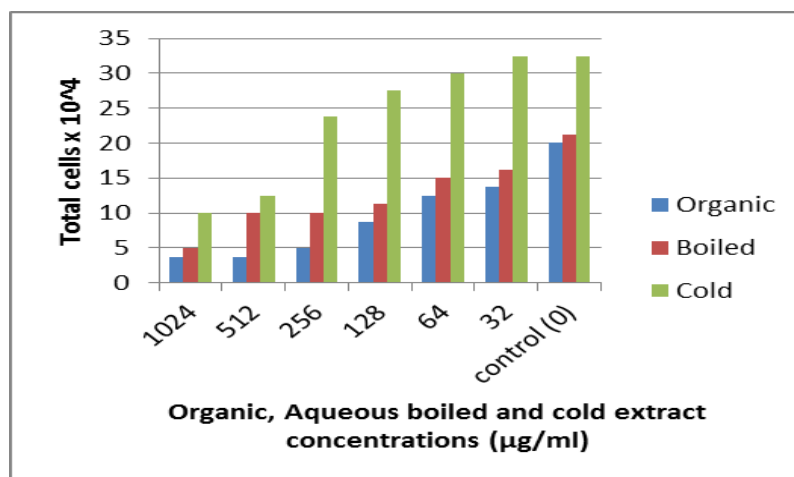


Figure 5: Cell count of Hela cell line after the application of *Arum palaestinum* three different extracts.

Although there was no clarification in literature explaining the reason why the hot aqueous extract gave better anti-cancer effects, it could be reasonable to speculate that the active ingredients with the anti-cancer activity are water soluble. Reports indicated that phenolic compounds including flavonoids are promising candidates for cancer prevention.^[16] However, this has to be further investigated using HPLC and GC to specifically identify the active effective components of each extract.

CONCLUSION

Arum Palaestinum crude extracts (organic, aqueous boiled and aqueous cold) prepared from the leaves exert variable in-vitro anti- cancer activity against three types of cancer cell lines with no antimicrobial or antifungal effects. Further investigations (isolation and identification) of the active compound(s) are needed for possible formulation of new therapeutic alternatives.

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