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Research Article

Phytochemicals screening, cytotoxicity and antioxidant activity of the *Origanum majorana* growing in Casablanca, Morocco

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Abstract

Origanum majorana is a plant from the *Lamiaceae* family. It is a medicinal plant used in traditional medicine in Morocco to treat various diseases. This work aims to determine the phytochemical composition of Marjoram, as well as to evaluate its cytotoxic effect on the cells of healthy subjects. All parts of the plant (roots, leaves, stems, etc.) were subjected to selective extraction with different solvents of increasing polarity (Diethyl ether, Dichloromethane, Ethanol, Methanol) using the rotary steamer. The yields obtained are respectively 1.34%, 4.57%, 9.98%, and 10%. The phytochemical tests carried out have detected the presence of polyphenols, tannins, flavonoids, terpenoids, sterols, saponins, and reducing sugars. In contrast, the absence of the family of cardiotonic alkaloids, quinones, and heterosides. *Origanum majorana L.* exhibited concentration-dependent inhibitory effects on 2,2'-diphenylpicrylhydrazyl (DPPH) with IC_{50} equal 2.308 mg/ml, related to the presence of the same content of polyphenols and flavonoids but with the lowest concentration of tannin content. The cytotoxicity of the hydro-ethanolic extract of Marjoram was evaluated by the MTT colorimetric method. However, the results obtained that the examined extract was devoid of cytotoxic activity, on the other hand, it induced cell proliferation. *O. majorana* has good potential to prevent diseases caused by the overproduction of free radicals, and that it can be used as natural antioxidant agents and cell proliferation dependant on concentration.

Introduction

For centuries, traditional medicinal plants have been used by humankind to cure disease. Nowadays, many researchers became interested to treat various disorders with those medicinal plants [1].

Origanum majorana L. is a medicinal plant of the *Lamiaceae* family, knowing as Zaatar in traditional Moroccan medicine [2]. This plant is distributed around the Mediterranean regions, Asia, and North Africa, in particular, Morocco, Algeria, Egypt, Spain, and Portugal [3]. *Origanum majorana* showed various biological activities such as allergies, fever, hypertension [4,5], respiratory infections [6], antidiabetic [7], painful menstruation, Kidney Yang deficiency, stomach ache, cough [8], rheumatism, headache, insomnia [9], also in intestinal antispasmodic [10]. Moreover, *Origanum majorana L.* exhibits a wide effect spectrum with antioxidant, antibacterial, antifungal, nephroprotective, anti-proliferative, anti-cancer activities [11-16]. These effects are mediated by the presence of bioactive compounds such as thymol, carvacrol, tannins, hydroquinone, sitosterol, cis-sabinene hydrate, limonene, terpinene, camphene, and flavonoids like diosmetin, quercetin, luteolin, and apigenin [17,18].

Recently, the excessive use of antibiotics was conducted

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to the development of antibiotic resistance and without a doubt, the incidence of multidrug-resistant pathogens is considered a major disadvantage in medication strategy, which has led attention towards innovative antibiotic sources. On the other hand, the plant had a therapeutic potential against drug-resistant microbial infections [19]. The herbal made four major groups of antimicrobial compounds like phenolics and polyphenols, terpenoids and essential oils, lectins and polypeptides, and alkaloids, those bioactive plant extracts could yield an enhanced effect (Cowan,1999). A lot of mechanisms against the bacteria were used via these compounds, including inactivation of proteins, adhesins, and enzymes, among other targets.

Some recent research revealed that certain molecules in the plant could also block cell-to-cell signaling pathways, extinguish the production of virulence factors and disrupt or inhibit the formation of biofilms, which bestow a protective advantage to pathogens during infection [20].

Nevertheless, *Origanum majorana* leaves wielded significant antimicrobial activity against a huge spectrum of Gram-positive and Gram-negative bacteria: Bacillus cereus, Escherichia coli, Staphylococcus coagulase-positive, Enterobacter spp, Proteus spp, Acinetobacter spp, Klebsiella spp., Pseudomonas spp S, aureus, Klebsiella pneumonia, and Pseudomonas spp. Besides, the sensitivity results obtained are almost the same as those observed for the antibiotic gentamicin considered as a positive control [10]

The purpose of the present study was to characterize the flavonoid, polyphenols, and tannins content, antioxidant properties, and cytotoxic activity of *O. majorana.*

Materials and methods

Plant material and extract preparation

Origanum majorana (*O. majorana*) was collected from Casablanca, Morocco between February and April. The plant was identified by a botanist. The collected plant was dried in the shade at room temperature for two weeks. One kg of the plant was powdered with an electric grinder, 20 g of the pulverized plant material was extracted in 200 ml of hydro-ethanol and hydro-methanol (80:20) separately. Another 20 g of plant material was extracted in 200ml of diethyl ether and dichloromethane separate. Then, th-e separated extracted were t filtered through Whatman's No. 1 filter paper. The excessive solvent was evaporated with a rotary vacuum evaporator using the boiling time of each solvent. The yield percentage was calculated using the following equation:

$$\text{The percentage yield of extrac} = \left(\frac{\text{Weight of extracted material}}{\text{Weight of original plant material used}}\right) x100$$

Humidity determination

Two g of *O. majorana* was placed in previously weighed glass capsules. The whole was placed in a vacuum oven at 105 ° C for 24 hours. Three repetitions were performed according to a previously reported method of Cunniff, 1997 [21]. When drying was finished, the humidity percentage was calculated according to the following formula:

$$Humidity\ (\%) = \frac{T2-T3}{T2-T1} X100$$

T1: the weight of the empty capsule, T2: the weight of the capsule containing the fresh sample, T3: the weight of the capsule containing the dry sample.

From the moisture content we can determine the dry matter content which is calculated by the following formula:

Dry matter content (%) = 100 - Humidity

Preliminary qualitative phytochemicals screening

The extract of the plant material was subjected to phytochemical screening to qualitatively determine some types of interested phyto-organic constituents which are responsible for biological activities, alkaloids, polyphenols, quinones, saponins, tannins, sterols, carbohydrates, glycoside cardiac, triterpenes, and terpenes which were the major cheeked groups using standard methods.

Determination of total phenolic content

Folin-Ciocalteu reagent method described by Zhishen, et al. 1999 [22] was used to detect total phenolic content (TPC) present in the hydro-ethanolic extract of *O. majorana*. A volume of 1.5 mL of 10% Folin-Ciocalteu reagent was added to 0.5 ml of extract and mixed for 5 min. Then, 3 mL of sodium carbonate solution 7.5% (Na_2CO_3) was added and further incubated at 30°C for 2 h. Finally, the absorbance was calculated at 760 nm using a UV-visible spectrophotometer against a blank composed of the same previously reagents except for the extract. The quantity of total phenolic compounds was detected from the standard curve of gallic acid and expressed in mg gallic acid equivalent (GAE) per g of the dry weight of extract (mg GAE/g DW).

Determination of total flavonoid content

Aluminum Chloride ($AlCl_3$) colorimetric method is described by Dewanto, et al. 2002 [23]. Was used to detect the Total Flavonoids Content (TFC). Briefly, one mL of 2% $AlCl_3$ solution was added to 1 mL of the hydro-ethanolic extract of *O. majorana* and incubated for 30 min. The absorbance was measured at 420 nm and the flavonoid content was detected from a quercetin standard curve and expressed in mg quercetin equivalents per g of the dry weight of extract (mg QuE/g DW).

Determination of condensed tannins content

Condensed Tannins Content (CTC) was determined according to the method of Sun, et al. 1998. An aliquot of the extract with different concentrations (0.5-2-4mg/mL) was mixed with 3mL of vanillin (4%) and 1.5 mL of sulfuric acid concentred. After homogenization, the tubes are incubated in darkness at ambient temperature for 15min.

Then, the absorbance was measured at 500nm. The standard curve was prepared by using different concentrations

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of catechin and the absorbance was measured on the same wavelength. Total condensed content was expressed as mg catechin equivalents of the dry weight of individual extract (mg CE/g DW).

Antioxidant activity

The DPPH free radical scavenging activity to hydro-ethanolic extract of *O. majorana* was quantified according to the method of Wu, et al. 2019 [24], with some modifications. Briefly, 50 µL of extract with different concentrations (0.5-2-4 mg/mL) was added to 1950 µL of 60 µM DPPH ethanol solution (2.3mg of DPPH in 100mL ethanol).The mixture was shaken strenuously and incubated 30 min at room temperature. Absorbance was recorded at 517 nm. The scavenging activity was calculated using the formula:

$$\text{DPPH scavenging activity}(\%) = \left[\frac{(\text{A0-AE})}{(\text{A0})}\right] \times 100$$

Where A_0 and A_E are the absorbance of the control and extract after 30 min, respectively.

PBMCs isolation and cell culture

The whole blood was collected from healthy adults informed with written consent. The PBMCs were isolated using Ficoll-Histopaque (d=1.077g/mL) (Sigma, USA), by centrifugation at 900g for 25 min at 20°C. After washing cells in NaCl 0.9% at 400g for 15 min and resuspended in RPMI-1640 media (Gibco, UK), enriched with 10% of Fetal Bovine Serum (FBS) (Sigma, USA), 26.3g/L penicillin, and 4.2g/L Streptomycin, the viable cell counting was performed by Trypan blue method using microscopy. 100µL of complete media containing $1x10^5$ cells were seeded in triplicate using 96 well plate culture, supplemented with 20 µL of different concentrations of *O.majorana*, and the final volume (200µL/well) was reached with complete media. Then, the PBMCs were incubated for 96 hours at 37°C under 5% CO2 and 95% humidity.

MTT assay

Viability and proliferation were measured by the MTT (3-(4,5 dimethyl thiazol-2-yl)-2,5 diphenyltetrazolium bromide) test [25] in each well and compared to that of the untreated group. After incubation, 20µL (5mg/mL) (Sigma, USA) was added to each well and the plates were incubated 4h at 37°C under 5% CO2 and 95% humidity.

Thereafter, the supernatant was removed from each well after centrifugation, and 100µL DMSO was added to dissolve the formazan crystals produced following the viable cells able to metabolize MTT salts. The extent of the formazan product was measured at 492nm using FLUO star Omega microplate reader.

Cell viability was calculated using the following formula:

Cell viability (%) = (mean of optical density of treated cells /mean of optical density of negative control cells) x100

And cell proliferation was calculated using the following formula:

$$\text{proliferationIndex} = \left[\frac{(\text{OD testsample- OD control})}{\text{OD control}}\right] \text{x } 100$$

Statistical analysis

The statistical significance between the mean values was performed by an independent t-test. using SPSS statistical software version (15.0.1) (Chicago, IL) to determine the difference between groups. All the [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$] were considered significant.

Ethics

The study was assessed and approved by the Faculty of Medicine and Pharmacy, Hassan II University, Ethics Committee, following the declaration of Helsinki. Consents were obtained for the collection of PBMCs samples.

Results

Extracts yields and humidity

The yield extracted from *O. majorana* by four solvents namely, hydro-methanol, hydro-ethanol, diethyl ether, and dichloromethane is presented in Figure 1. The highest extract yield was obtained by hydro-methanol extraction followed by hydro-ethanol, dichloromethane, and finally by diethyl ether. The yields of both hydro-methanol and hydro-ethanol were 9.98 and 10 % respectively. Diethyl ether showed as a solvent was lower than the efficiencies of all other solvents.

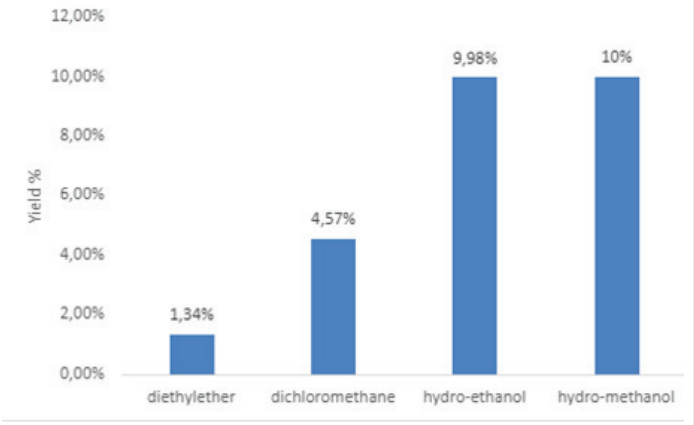

Figure 1: Percentage of yield extracts of O. majorana

Although, *O. majorana* exhibited the lowest humidity percentage with a value of 10.107 ± 0.25%. then, the water content allowed to determine the percentage of dry matter estimated at 89.893%.

Phytochemicals screening

The preliminary phytochemicals of O. majorana revealed the presence of important secondary metabolites such as tannins, polyphenols, flavonoids, and saponin. Carbohydrates,

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terpenoids, sterols, and triterpenoids were found in moderate concentration while quinone and glycosides cardiac was not found. Although, the alkaloids were present by the Mayer test but not by Dragendorff (Table 1).

Determination of total polyphenols, flavonoids, and tannins content

Based on the absorbance values of the hydro-ethanolic extract solution, which reacts with the Folin-Ciocalteu reagent and compared to standard solutions of gallic acid equivalents ($y=0.0064x - 0.1213$, $R^2=0.9288$) a polyphenol content of (3.87 to 5.15 mg GAE/g DW) was found (Table 2).

The result of flavonoids content revealed their presence of a concentration between 3.02 to 5.62mg QuE/g DW this result was differentiated with a standard curve of quercetin. The flavonoid contents were calculated using the equation $y=0.0352x+0.0273$, $R^2=0.9889$.

While the number of condensed tannins varied by increasing concentration in the hydro-ethanolic extract of *O. majorana* and ranged from 1.9 to 4.4 mg CE/g of dry material (Table 2). Catechin was used as a standard compound and the condensed tannins content were expressed as mg/ml catechin equivalent using the standard curve equation: $y = 0.003x + 0.021$, ($R2= 0.995$).

Antioxidant activity

The radical Scavenging activity of hydro-ethanolic extract of *O. majorana* was examined using the DPPH radical at different concentrations. Scavenging activity against the DPPH radical was concentration dependant. The IC_{50}, the concentration at which 50% of DPPH molecules are inhibited, is obtained from the point of intersection of the scavenging activity and the antioxidant activity curves (Figure 2). IC_{50} corresponds to 2.308 mg/mL of *O. majorana.* The maximum scavenging activity of the hydro-ethanolic extract was 53.068% at 4 mg/mL (Table 2).

Table 1: Phytochemicals screening of *Origanum majorana.*

phytochemical compound		Presence / absence
Tannin	Catechic	+++
	Gallic	+++
Polyphenols		+++
Flavonoids		+++
Quinone		-
Alkaloids	Mayer	+
	Dragendroff	-
Terpenoids		++
Sterols and triterpenoids		++
Saponin		+++
Glycosides cardiac		-
Carbohydrates		++

+++= presence maximum, ++= moderate presence, +=minimum presence, -= absent presence

Table 2: Total phenolics, Flavonoids, Condensed Tannins content, and DPPH Scavenving activity of *O.majorana* extract.

Concentration	TFC (mg QuE/g DW)	TPC (mg GAE/g DW)	CTC (mg CE/g DW)	Scavenging activity (%)
0.5 mg/mL	3.02±0.05	3.87±0.01	1.9±0.009	45.90±0.08
2 mg/mL	4.68±0.07	4.6±0.04	3.48±0.008	47.72±0.01
4 mg/mL	5.62±0.05	5.15±0.06	4.44±0.02	53.068±0.04

Cytotoxicity assay

MTT assay investigated the effect of hydro-ethanol extract of *O. majorana* on PBMCs viability. A dose-dependent MTT reduction was observed by a color change from yellow to purple. After 5 days of application, *O. majorana* showed a statistically significant increase in the percentage of cell viability between the medium and the concentrations of 300 and 600 µg/mL. Whereas the comparison with the concentration of 150 µg/mL showed only a slight increase. This means *O. majorana* extract increases the viability of immune cells (Figure 3).

However, the cut off showed in Figure 4 at which the extract induces a proliferative effect is set at 0.392. At a concentration of 150 µg/mL, the proliferation index is below the positivity threshold, which means that at this concentration the extract

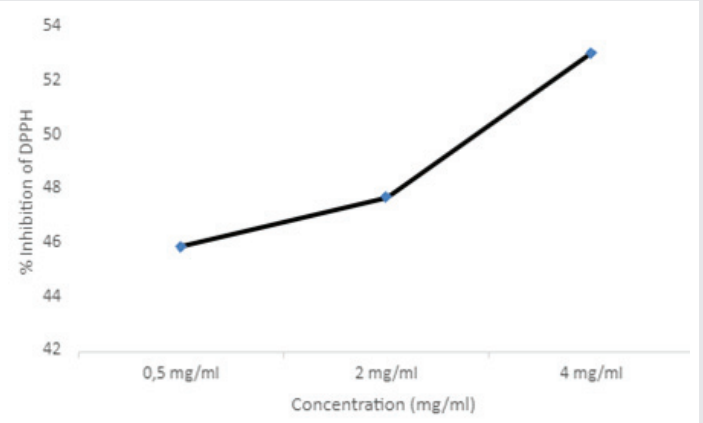

Figure 2: DPPH scavenging activity of O. majorana extract.

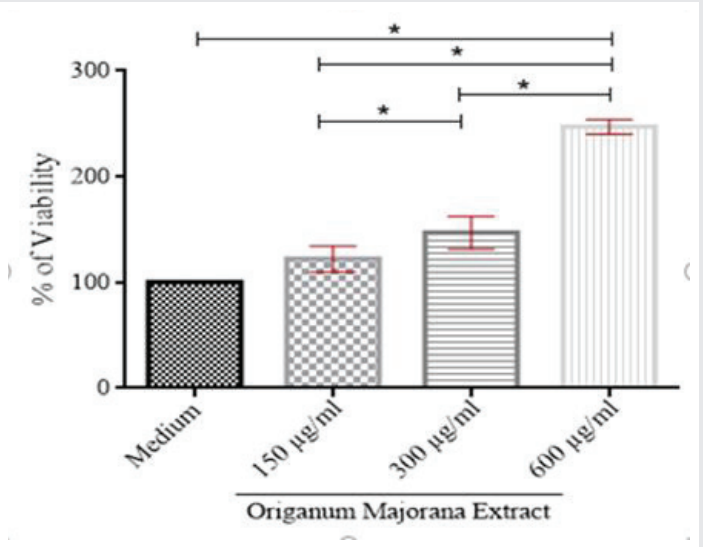

Figure 3: Percentage of viability cells of *O. majorana*.

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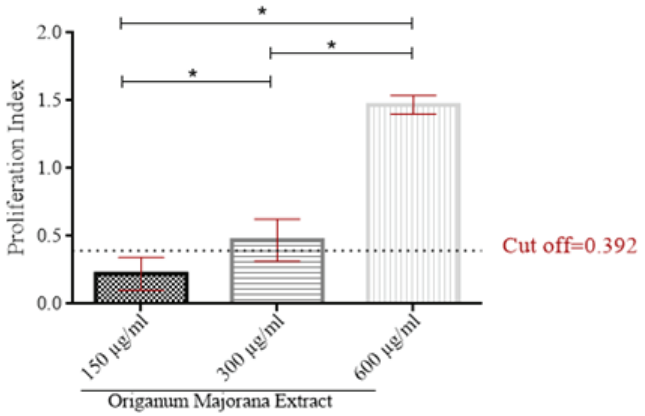

Figure 4: Proliferation index of O. majorana extract.

of *O. majorana* has no proliferative effect, while at 300 and 600 µg/mL the proliferation index is statistically higher than the positivity threshold when the concentration increases, this being said that from 300 µg /mL the extract showed a proliferative effect.

Discussion

Recently, medicinal plants are known for their various biological activities to cure humans against many diseases. Their activities were related to the presence of secondary metabolites such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumins, saponins, phenolics, flavonoids, and glucosides [26].

The extraction method adopted is successive exhaustion using four solvents of increasing polarity. The best yields are obtained with the more polar solvents, namely hydro-methanol and hydro-ethanol, while the lowest yields have been obtained with dichloromethane and diethyl ether, which are less polar solvents.

This difference of yield could be first caused by the physicochemical characteristics of the solvents used, in particular on their polarity. Secondly, according to several studies, geographical origin of species, drying time, as well as the extraction method, and conditions such as pH and temperature are among other factors that may also have a direct impact on extraction yields, which makes it difficult to compare our results with those found in other studies [27].

Generally, the plants are rich in water while the analysis of our sample revealed a low humidity rate of 10%, this low rate reflects good conservation of the powder therefore very low degradation of the bioactive molecules of the plant.

Our results are a little high with those of Shahidi, et al. 2018 [28] who stated that the moisture content of *O.majorana* is between 6.5 and 8%. Numerous studies have shown that the humidity level is only relative can be related to environmental factors such as climatic conditions, geographical origin as well as the conditions, methods, and applied drying time.

The preliminary assessment of the phytochemical composition of the *O. majorana* species revealed the presence of secondary metabolites. Indeed, the results of the phytochemical screening revealed the presence of polyphenols, flavonoids, catechin, and gallic tannins, terpenoids, sterols, and triterpenes as well as saponin and carbohydrates, while other compounds seemed to be absent in our sample, in particular, glycoside cardiac, alkaloids, quinones, and heterosides. Our result is following those of Adam, et al. [29] and similar to those of Bhardwaj, et al. [30].

However, Vasudeva reported the absence of alkaloids and glycosides in this species, which is comparable to our results [31]. Another study carried out on the methanolic extract of *O. majorana* in Yemen confirms the results of our work by confirming the absence of alkaloids and the presence of other chemical compounds [32]. Contrariwise, the literature has shown the existence of various alkaloid molecules while they were not detected in our plant sample. This disagreement on the chemical composition of the same species can be justified by differences in geographical location and extraction method [33].

The choice of using hydro-ethanol extract was because the ethanol was a better solvent than the others in extracting phenolic compounds from the extracts due to his polarity and good solubility for phenolic components from plant materials [34].

Under our study, we found a slight variation between total phenolic and flavonoids content in the three concentrations while a low concentration of tannins (Table 2). That was enabled the correlation between the total flavonoid, polyphenols content, and antioxidant activity. Our results are in an agreement with those [35] and disagreed with [36]. We suggest that could be concerning high levels of polar compounds in the plant materials which are soluble in solvents with a high polarity such as water, methanol, or ethanol.

The determination of the antioxidant activity of *O.mjorana* extracts was based on the scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. DPPH radicals are widely used for the preliminary screening of compounds capable of scavenging activated oxygen species [37]. Ascorbic acid was used as a standard.

In this study, the extract of *O. majorana* exhibited IC_{50} values for the radical scavenging activity was found to be 2.308 mg/mL, this activity could be associated with phenolic compounds and how they played an important role in stabilizing lipid peroxidation. Previous work revealed that *O. majorana* essential oils and ethanolic extract exhibited high antioxidant activity, also water, methanol, and chloroform extracts [38].

The cytotoxicity and cell viability of the hydro-ethanolic extract of *O. majorana* species to human PBMC from healthy donors was carried out using an in vitro (MTT test), which is known for specificity, sensitivity, and reliability [39]. Our data revealed that doses (150, 300, 600 µg / mL) of *O. majorana* extract exhibited a stimulating effect on the cell viability

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of human PBMCs, resulting in a significant increase in cell proliferation from the concentration 300 µg/mL. This increase in cell viability shows that the extract has no toxicity effect on human cells, on the contrary, it potentially modulates the cellular immune response. RPMI did not affect PBMCs; therefore, the effect observed will be that of the extract used. Our results are consistent with an Indian study evaluating the effect of ethanolic, methanolic, and aqueous extract of *O. majorana* on normal lymphocytes at a concentration range of 40 to 120 µg / mL. The ethanolic and aqueous extracts were found to be non-cytotoxic on peripheral blood lymphocytes, while the methanolic extract was slightly toxic [14].

Besides, the cytotoxic and antiproliferative effect of extracts of *O. majorana* on human cancer cell lines of the breast and colon was elucidated by a Moroccan study where concentrations of extracts ranging from 15.6 to 500 µg / mL induced DNA damage, growth arrest, and apoptosis, resulting in a considerable decrease in cell viability [40].

The anticancer potential of *O. majorana* has also been shown by other studies that have shown inhibition of dose-dependent proliferation of human cancer cell lines such as fibrosarcoma, leukemia, lung cancer cells, larynx, and carcinomas. hormone-dependent prostate [10,14,41]. An Indian study has suggested that *O. majorana* has particularly anti-tumor biological properties on cancer cells seem attributed to the chemical composition of the plant which is rich in polyphenols, flavonoids, and tannins. P-coumaric acid has proven its antiproliferative effect against colon cancer cells by causing mitochondrial disturbances and apoptosis in these cells [42-46].

Conclusion

In conclusion, *in vitro* efficacy of the hydro-ethanolic extract of *Origanum majorana* in the proliferation of the cells. Theis herbs contain high phenolic and Flavonoid compounds that showed antioxidant and free radical scavenging activities. More studies will be required to purify and isolate the bioactive fractions of this extract also the effect on the immune system.

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