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Anti-Cancer Effect of Mentha pulegium L.

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ABSTRACT

Background: Many chemotherapy drugs used for treatment cancer, but has many side effects. A lot of studies assessed natural extract as anti-cancer agent and reduced cells proliferation.

Objective: The present study was done to investigate the anti-cancer effect of M. pulegium leaves ethanol extract fractions and investigate the active component by GC-MS spectrum analysis. **Methods:** Cytotoxicity and anti-cancer ability of M. pulegium extract was investigated by exposing tumor cell line (MDA-MB-231, MCF7 & PC3) and Human hepatic cell line (WRL68) to different doses from M. pulegium extract. We evaluated the ability of MTHF2 in induction apoptosis process in the treated cancer cell by using AO/EB stain for estimation of apoptotic cells.

Results: M. pulegium extract was no toxic effect on the WRL68 while decreasing in the viability percent of treated MCF7, MDA-MB-231 and PC3 cell in doses dependent manner, lost their normal shape to be around, detached floating in culture media with reduction in the number then become under growth inhibition. Alteration in the nuclei of the normal cell was clearly showed by Fluorescent microscope image to cells stained with double AO/EB stain, which nuclei of apoptotic cell appeared fluorescent green, condensed chromatin, DNA fragmented with formation of apoptotic bodies. MTHF2 was applied to GC-MS analysis for diagnose the active compounds, identified twenty two active compounds; the most abundant are Ethyl Oleate in the percentage of 62.68% and Octadecanoic acid, ethyl ester in the percentage of 66.06%. Both compounds have a high antioxidant effect. **Conclusion:** M. pulegium leaves extract fraction 2 has anti-cancer effect by inhibition the cancer cell proliferation throughout enhanced apoptosis process.

Keywords: Anti-cancer, Apoptosis, Mentha pulegium L., Anti-proliferation, Experimental study, University of Diyala.

INTRODUCTION

Mentha pulegium L. is one of the important and have many used in traditional medicine. The native area is Eastern Mediterranean, but distributed in different countries throughout the world. It is used as spices add to meat or drink as tea. M. pulegium leaves tea used in traditional medicine in Iraq in the treated bowel syndrome, relaxing, and reducing menstrual pain. Many previous studies go throughout M. pulegium to investigate the biological properties and chemical components. M. pulegium leaves methanol extract was investigated for antibacterial activity and the result was showed a high effect on gram- positive and gram - negative bacteria [(1)].

The essential oils from M. pulegium showed bacteriostatic and bactericidal activity [(2)]. Other previous studies extract chemical nanoparticles such as Silver, Bismuth Oxide, Zinc Oxide and Copper from M. pulegium leaves extract [(3-5)] and from another medicinal plants as antioxidant and anti-immunosupression [(6)]. The medicinal plants used in the medicine application as anti-inflammation in wound healing[(7)]. M. pulegium leaves extract have highly total phenolic and flavonoid content with high antioxidant properties [(8)]. The HPLC analysis for M. pulegium leaves extract was indicated for present many active compounds with biological activity [(9)]. Also, GC-MS spectrum analysis was showed the existence of many compounds that important in the pharmacological and industry purpose [(10)]. The present study was done to investigate the anti-cancer effect of M. pulegium leaves ethanol extract fractions and investigate the active component by GC-MS spectrum analysis.

METHODS

Chemicals: Ethanol was purchased from Fisher Scientific, UK. PBS buffer from Dulbecco A, Oxoid, England. Silica gel G60, 70-230 mesh from Merck, Darmstadt, Germany. WRL 68 cell line, MCF7 cell line, MDA-MB-231 cell line and PC3 cell line (American type culture collection, ATCC, Rock-ville, MD).DMEM media by Sigma-Aldrich, UK. Fetal bovine serum by Biowest, France. DMSO by Fisher Scientifc, UK. Acridine Orange (AO) and Ethidium Bromide (EB) were purchased from Fisher Scientific, UK.

Extraction of plant and fractionation: Leaves of M. pulegium (voucher number EM4.12) were purchased from the native market then cleaned, left to air- dry. After that made powder by using an electric blender and it is applied for extraction and fractionated as described previously by ***Abood et al.*** [(11)].

M. pulegium cytotoxicity and anti-cancer ability: The cytotoxicity and anti-cancer ability of M. pulegium leaves extract was investigated by exposing tumor cell line (MDA-MB-231, MCF7 & PC3) and Human hepatic cell line (WRL68) to different doses at final concentration 125, 250, 500, and 1000 µg/mL from M. pulegium leaves

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extract. For the anti-cancer effect for M. pulegium leaves extract fraction was evaluated throughout treated MDA-MB-231, MCF7 and PC3 cells with doses at final concentration 125, 250, 500, and 1000 µg/mL. The method was performed as previously described in ***Abood, et al.*** [(11)].

Apoptosis mechanism induced by M. pulegium: To evaluate the ability of M. pulegium in induction apoptosis process in the treated cancer cell, which lead cell to programmed cell death. The method was performed upon the cell MDA-MB-231, MCF7 and PC3 cells reached to confluent 80 to 90% harvested the cell, count 1 X 10^5 cell/ mL, 100 µL (5000 cell/well) was suspended in the 96 well plate from each cell and incubation at 37ºC and 5% CO2 for 24 hours, then culture media was removed from the well and replaced with 100 µL from fresh media contains different doses from M. pulegium fraction at a final concentration 25, 50, 100, and 200 µg/mL. Treated cells were incubated for 24 hours at 37ºC and 5% CO2. The control is well contains cells with only DMEM media. After 24h was ended, 10 µL from MTT solution added to all wells, incubation for 4h at 37ºC. The remain media and MTT aspirated then 100 µL of dimethyl sulfoxide was added for dissolving the MTT tetrazolium salt and developed purple MTT formazan salt. The amount of this salt was measured by absorbance at 570 nm wave length; the density of purple color was proportional to the viable cells.

Estimation of apoptosis: To estimate apoptosis in the treated cells (MDA-MB-231, MCF7 and PC3), the cells dealt with AO/EB stain. After treating MDA-MB-231, MCF7 and PC3 cell with M. pulegium fraction for 24 hours at 37ºC, media was removed then cells were washed with PBS at (pH 7.4) then 100 µL from equal percentage AO/EB stain was added and incubated the plate in the dark place at 37ºC for 30 min. The estimation of apoptotic cells was detected by a fluorescent microscope under FITC filter at 20X magnification [(12)].

Investigate active constituents in M. pulegium leaves Fraction: M. pulegium leaves Fraction was applied to Gas chromatography mass spectrum (GC-MS) that done by MSDCHEM instrument for detecting the phytochemical constituents for the M. pulegium leaves Fraction. The methods was performed by 0.5 µL from fraction was injected, the 1:50 split ratio, 250 temperature used as injection temperature and back an eluent temperature, the column flow 1mL/min. helium is used as carrier gas. The oven temperature was 60-246; the rate 4C/minutes. Active compounds were identified by compared the detected compound spectrum to the compounds in the Wiley and NIST/EPA/NIH mass spectral libraries.

Statistical Analysis:

Quantitative data was expressed as mean ± standard deviation (SD). ANOVA test was used for comparison between more than 2 groups and the significant of variance at P value ≤0.05. Image J software used for analysis of AO/EB picture to estimate apoptosis.

RESULTS

Cytotoxicity of M. pulegium: The results were showed that M. pulegium leaves ethanol extract no toxic effect on the Human hepatic cell line (WRL68), the viability of treated WRL68 cells with doses 125, 250, 500, and 1000 µg/mL was 76.94 ± 0.85, 78.13 ± 1.00, 78.6 ± 0.73 and 88.39 ± 1.46, respectively, while decreasing in the viability percent of treated human cancer cell line (MCF7, MDA-MB-231 and PC3 cell) significantly (P<0.05) in doses dependent manner compared with WRL68 cells. The viability percent of MCF7 cell was 54.23 ± 45.77, 56.49 ± 43.51, 58.04 ± 41.96, and 63.11 ± 36.89, for MCF7, MDA-MB-231 was 56.71 ± 1.57, 50.19 ± 0.17, 48.83 ± 1.41, and 45.85 ± 5.59 and for PC3 cell was 29.38 ± 0.59, 28.80 ± 0.79, 25.77 ± 0.14, and 24.51 ± 0.84, respectively to doses (**Figure 1**).

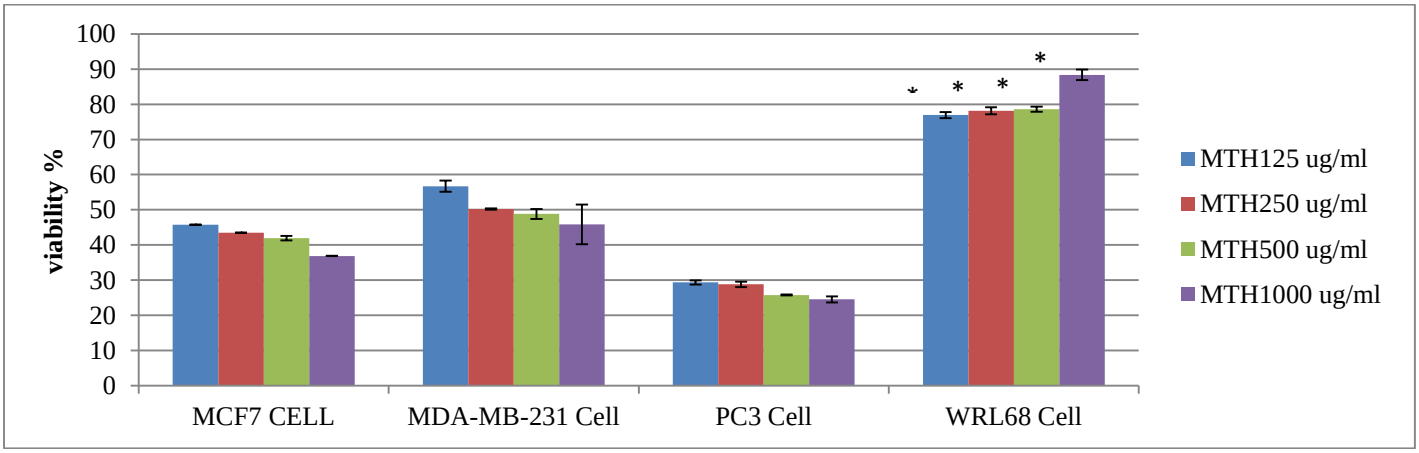

Figure 1: Viability percentage effect of M. pulegium leaves ethanol extract (MTH) on the Human hepatic cell line (WRL68) and human cancer cell line (MCF7, MDA-MB-231 and PC3 cell) with treatment doses 125, 250, 500, and 1000 µg/mL. Data was presented as mean percent ± SD, *Significantly (P<0.05).

Anti-proliferation activity of M. pulegium leaves fraction:

For proofing, the anti-cancer effect of M. pulegium leaves fraction the anti-proliferative ability investigated to M. pulegium leaves fraction on the human cancer cell line (MCF7, MDA-MB-231 and PC3 cell). The results were showed that M. pulegium leaves all fractions no toxic effect on the Human hepatic cell line (WRL68), the hexane fraction (MTHF2) gave the highest viability percent of treated WRL68 cells with doses 125, 250, 500, and 1000 µg/mL, 100.01 ± 2.12, 100.45 ± 2.12, 105.33 ± 0.94 and 123.32 ± 1.00, respectively, while all fractions reducing the viability of treated human cancer cell line (MCF7, MDA-MB-231& PC3 cell) significantly ($P<0.05$) in doses dependent manner compared with WRL68 cells. The MTHF2 gave highest inhibition percent of MCF7 cell was 29.26 ± 0.74, 44.22 ± 1.16, 68.16 ± 0.05, and 77.82 ± 0.72, for MCF7, MDA-MB-231 was 61.34 ± 8.89, 73.29 ± 0.78, 74.58 ± 0.53, and 76.33 ± 0.54 and for PC3 cell was 77.06 ± 0.23, 79.29 ± 0.18, 82.52 ± 0.31, and 83.38 ± 0.44, respectively to doses (**Figure 2**).

The result of this work was proven that M. pulegium leaves hexane fraction (MTHF2) has anti- cancer and anti-proliferation effect on the human cancer cells (MCF7, MDA-MB-231 amd PC3 cell) significantly ($P<0.05$) in doses dependent manner compared with less cytotoxicity effect on the WRL68 cells (**Figure 2**).

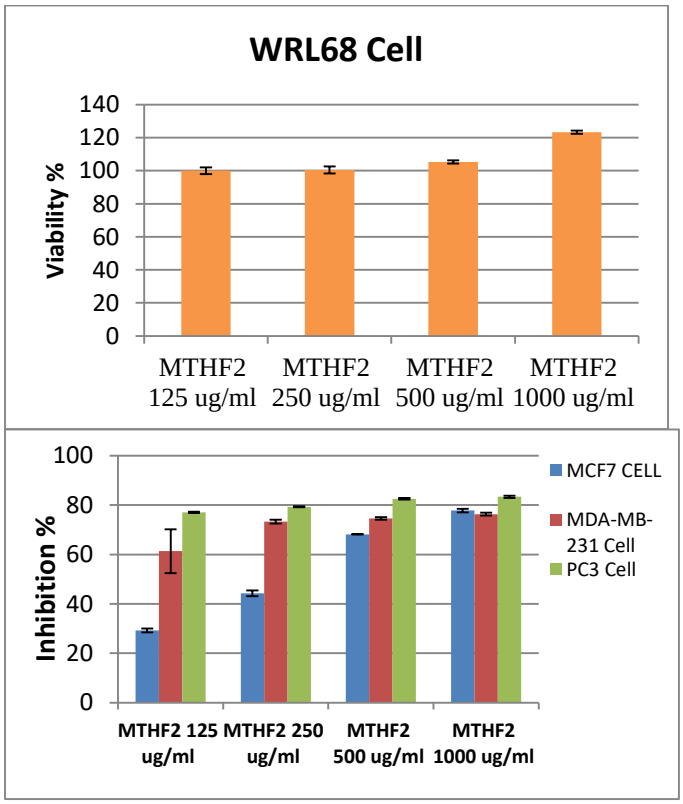

Figure 2: Viability percentage effect of M. pulegium leaves extract fraction 2 (MTHF2) on the Human hepatic cell line (WRL68), and inhibition percentage of human cancer cell line (MCF7, MDA-MB-231& PC3 cell) the with treatment doses 125, 250, 500, and 1000 µg/mL. Data was presented as mean percent ± SD, *Significantly ($P < 0.05$).

Alteration in the morphology of the cell:

Morphological alteration in the treated cancer cells with MTHF2 was shown in **Figures 3**. Morphological alteration is very clear in the treated cells compared with non-treated cells that keep normal shape and attachment with confluent between 95-100%, whereas treated cells (MCF7, MDA-MB-231 and PC3 cell) lose their normal shape be around and detached floating in culture media with a reduction in the number at dose deadened manner then become undergrowth inhibition.

The alteration in the nuclei of the normal cell was clearly showed by Fluorescent microscope image to cells stained with double AO/EB stain, which nuclei of apoptotic cell appeared fluorescent green, condensed chromatin, DNA fragmented with formation of apoptotic bodies (**Figure 4**).

Macrophage can rapidly recognize apoptosis cells and removed them before triggering inflammation, for that induced apoptosis was an important mechanism for chemotherapy drugs for cancer. For determination, if the cells inhibition and prevent cellular proliferation because of apoptosis process, for that assessed the MTHF2 with double AO/EB stain to investigate the morphological change and induction apoptosis with recognize apoptosis cells with its stages (**Figure 4**).

The results obtained by double AO/EB stain to diagnosed apoptotic cells were showed the percentage of apoptotic cells in the treated cells (MCF7, MDA-MB-231 and PC3 cell) with 200 µg/mL of M. pulegium leaves extract fraction 2 was significant ($P<0.05$) higher than in untreated cells and the morphological changing was presented in **Figure 5**, the percentage of apoptotic cells for untreated and treated MCF7 cells was 19.5 ± 12.58 and 63.41± 17.57, respectively, for untreated and treated MDA-MB-231cells was 16.03 ± 11.85 and 47.09 ± 13.10, respectively and untreated and treated PC3 cells was 17.11 ± 18.92, 36.1 ± 21.0, respectively.

Active compounds contents:

To diagnostic the active compounds content in the M. pulegium leaves extract fraction 2 (MTHF2), that responsible for the anti-cancer activity. MTHF2 was applied to GC-MS analysis for diagnose the active compounds. The result was identified twenty two active compounds (**Table 1**).

Table 1: The GC-MS analysis for active M. pulegium leaves extract fraction 2.

RT	Component	Formula	Area %	Biological Activity
15.22	Dichloroacetic acid, tridecyle…	C15H28C12O2	1.06	Anticancer [13]
15.22	Trichloroacetic acid,dodecyle...	C14H25C13O2	1.06	Anticancer [13]
15.60	(E)-Hexadec-2-enal	C6H10O	1.91	Antimicrobial [14]
15.60	(E)-1-(Methoxymethoxy)1tetrad...		1.91	Not mentioned
15.60	(E)-Tetradec-2-enal	C14H26O	1.91	Antioxidant, antimicrobial,anti-inflammatory [15]
18.36	Bromoacetic acid, hexadecylester	C18H35BrO2	0.44	Antioxidant, antimicrobial,anti-inflammatory [16]
21.53	Hexadecanoic acid, ethyl ester	C18H36O	16.06	Antioxidant & antidiabitic [17]
23.23	Octadecenoic acid, methyl est...	C19H36O2	1.17	Antimicrobial[18]
24.20	Ethyl Oleate	C20H38O2	62.68	Antioxidant [19]
24.52	Octadecanoic acid, ethyl ester		66.06	Antimicrobial [18], Antioxidant [20]
24.96	1,9-Cyclohexadecadiene	C16H28	2.49	Antioxidant, Antimicrobial [21]
24.96	Sulfuric acid,5,8,11heptadeca...	C18H32O3S	2.49	Antioxidant & Antidiabitic [17]
24.96	8,11,14-Eicosatrienoic acid,	C20H34O	2.49	Pro and anti-inflammatory [22]
27.28	Eicosanoic acid, ethyl ester	C22H44O	1.56	Antimicrobial [23]
27.28	Methyl 19-methyl-eicosanoate	C22H44O2	1.56	Antimicrobial [24]
27.28	Ethyl 14-methyl-hexadecanoate	C18H36O2	1.56	Antioxidant, Antimicrobial [25]
28.04	Tricosyl pentafluoropropionate	C26H47F5O2	1.30	Antibacterial [26]
28.04	Methyl 7,9-tridecadienyl ether	C14H26O	1.30	Antimicrobial [27]
29.59	Bis(2-ethylhexyl) phthalate	C24H38O4	3.22	Antimicrobial [28]
29.59	Diisooctyl phthalate	C24H38O4	3.22	Antimicrobial [29]
29.93	Docosanoic acid, ethyl ester	C24H48O	0.54	Antioxidant & antidiabitic[17]
29.93	Ethyl 14-methyl-hexadecanoate	C19H37O2	0.54	Antimicrobial [18]

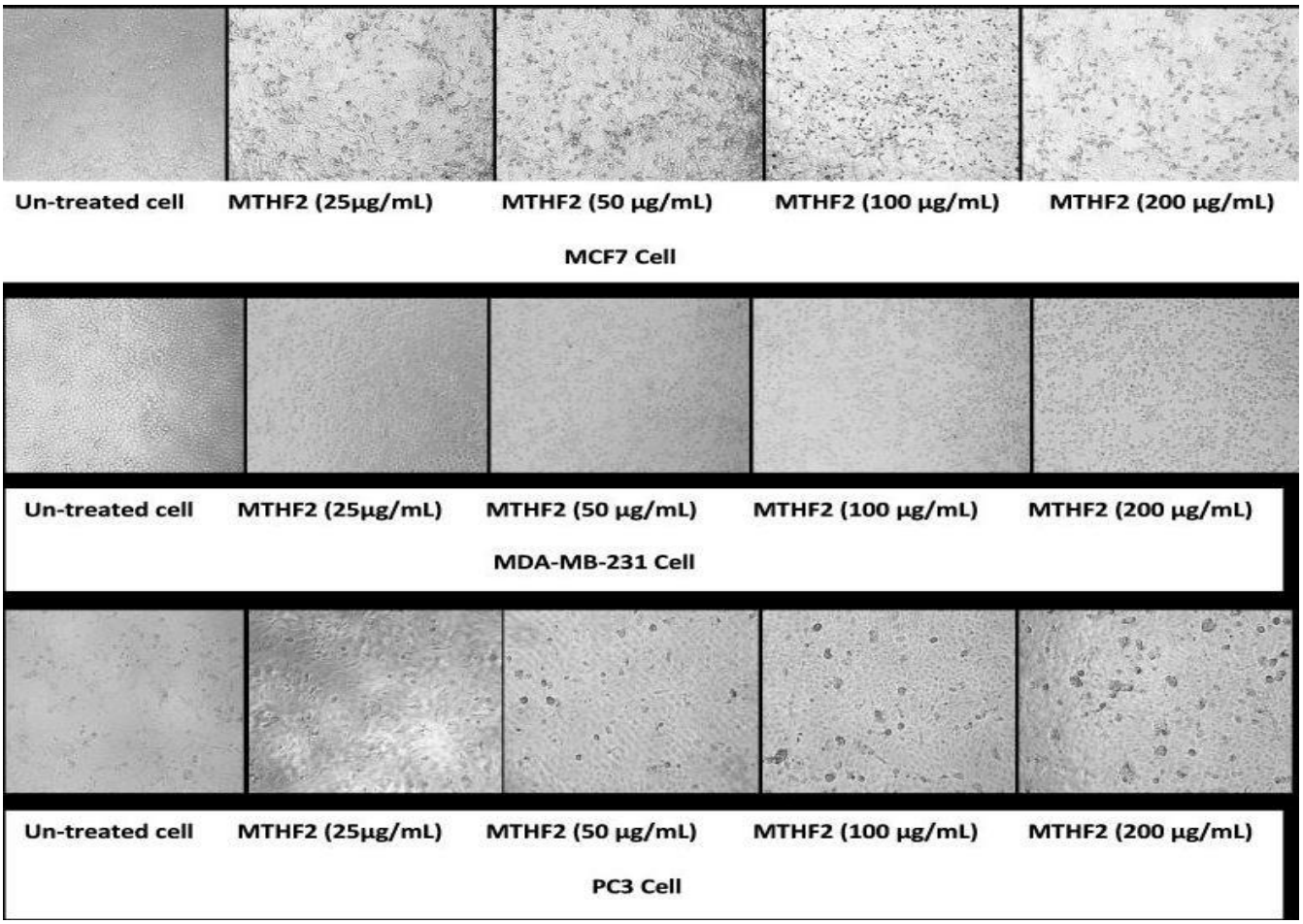

Figure 3: Morphological alteration in the treated cancer cells (MCF7, MDA-MB-231& PC3 cell) with different doses from M. pulegium leaves extract fraction 2 (MTHF2).

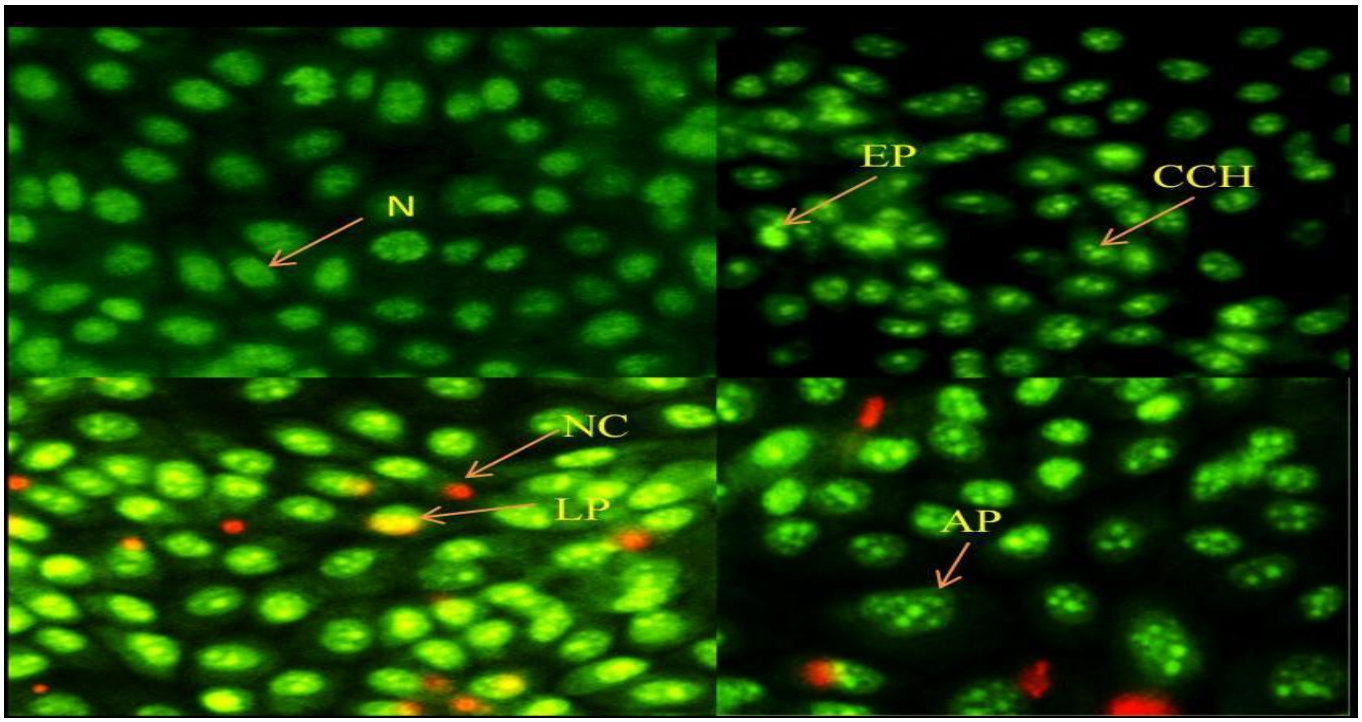

Figure 4: Morphological change companied to apoptosis in the treated cells with M. pulegium leaves extract fraction 2 stained with double stain AO/EB. N: untreated cell, EP: early apoptosis, CCH: condensed chromatin, LP: late apoptosis, NC: necrosis and AP: apoptosis bodies. Fluorescent microscope image at 400X.

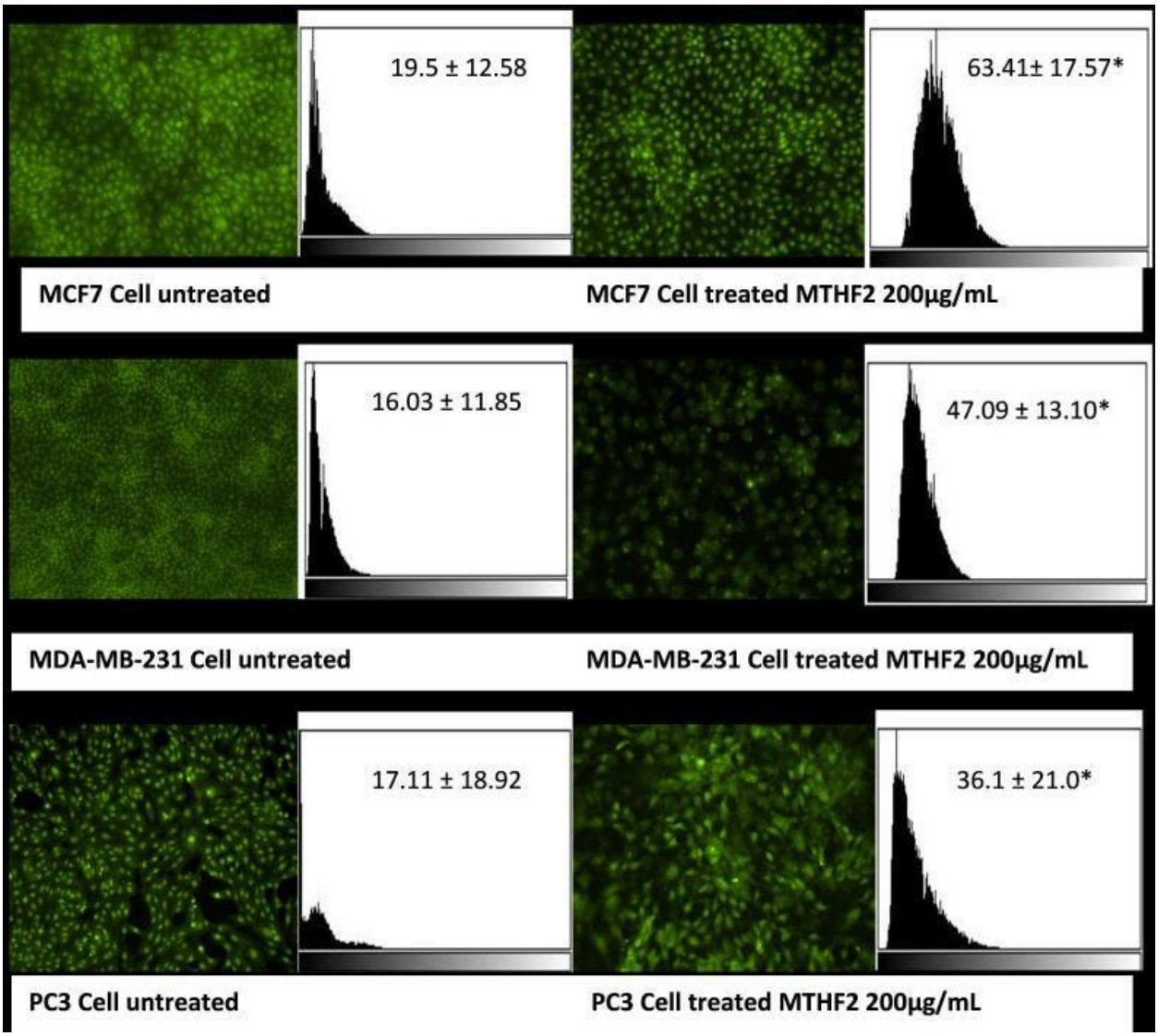

Figure 5: Presented results obtained by double AO/EB stain to diagnosed apoptotic cells with the percentage of apoptotic cells in the treated cells (MCF7, MDA-MB-231& PC3 cell) with 200 µg/mL of M. pulegium leaves extract fraction 2(MTHF2).
*Significant (P<0.05).

DISCUSSION

A third cause of death in the world is Cancer. There is much chemotherapy drugs used for treatment cancer, but it is has many side effects. A lot of previous studies were assessed natural extract as anti-cancer agent and reduction cells proliferation [30] by induction the apoptosis process and enhanced expression apoptosis genes, leads to reduction cancer proliferation and restriction tumor metastases caused cancer cell death [31] with less cytotoxicity and side effects [32]. The result of this work was proven that M. pulegium leaves hexane fraction (MTHF2) has anti-cancer and anti-proliferation effect on the human cancer cells (MCF7, MDA-MB-231 & PC3 cell) significantly (P<0.05) in doses dependent manner compared with less cytotoxicity effect on the WRL68 cells.

Natural bioactive compounds could modified and interfere with cardinal cellular action as inflammation, cell cycle, angiogenesis, apoptosis, metastasis and invasion [33]. An important process to destruct and prevent cellular proliferation is apoptosis, which is crucial for embryological development, cell differentiation, cell proliferation, and removal dead earnest damage cells, tumor cells [34]. Macrophage can rapidly recognize apoptosis cells and removed them before triggering inflammation, for that induced apoptosis was an important mechanism for chemotherapy drugs for cancer. For determination, if the cells inhibition and prevent cellular proliferation because of apoptosis process, for

that assessed the MTHF2 with double AO/EB stain was indicated the morphological change and induction apoptosis with recognize apoptosis cells with its stages in the treated cancer cell, that leading the cell to death and reduction cell proliferation with decrease cell count.

To diagnostic the active compounds content in the M. pulegium leaves extract fraction 2 (MTHF2), that responsible for the anti-cancer activity. MTHF2 was applied to GC-MS analysis for diagnose the active compounds. The result was identified twenty two active compounds. All components were identified in the previous studies have a biological activity as presented in (**Table 1**) such as antimicrobial, anti-oxidant, antidiabetic, anticancer with anti-inflammatory and proinflammatory effect. These abilities made the MTHF2 has the powerful as anti-cancer effect by induction of apoptosis through its content of Ethyl Oleate in the percentage of 62.68% and Octadecanoic acid, ethyl ester in the percentage of 66.06%. Both compounds have a high antioxidant effect [(19,20)] caused the oxidative effect on cancer cells by decreasing the level of expression of proliferation genes as Bcl-2 and increasing the expression of apoptotic enhancement genes as Bax, caspase leads to toll-like receptor 4 (TLR4) reduction inflammation genes and proteins [(35)]. So, antioxidant or reactive oxygen species compounds (ROS) are fundamental mediators of many biological processes such as phagocytosis and detoxification reaction and apoptosis which clear cancer cells.

CONCLUSION

This study was demonstrated that M. pulegium leaves extract fraction 2 has significant anti-cancer effect for MCF7, MDA-MB-231 and PC3 cell line by inhibition the cancer cell proliferation throughout enhanced apoptosis process. The GC-MS analysis was identified that M. pulegium leaves extract fraction 2 have many bioactive compounds have antioxidant and anti-tumor properties.

Declaration of Interest: The authors declared there is no competing of interest

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