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Synergistic anticancer effect of combining metformin with olive (*Olea europaea* L.) leaf crude extract on the human breast cancer cell line MCF-7

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Abstract
Breast cancer is one of the most prevalent cancers among women globally. Several *in vitro* studies have shown that Metformin and Olive (*Olea europaea* L.) Leaf extract have anticancer activity when applied separately. This study investigates the impact of combining Metformin and olive leaves extract on MCF-7 cancer cells. Extraction executed with Soxhlet using different solvents. Viability of MCF-7 cells following treatments were determined using MTT. Combinations were quantified using Chou and Talalay method. Expressions of *TP53*, *BAD*, *BAX*, *CASP8*, and Tristetraprolin were quantified using qPCR. Results show that various combination ratios elicited better antitumor effects on MCF-7 than singly. qPCR results show that Metformin acts through p53-independent apoptosis whereas Olive Leaf crude extract may have a different killing mechanism. In conclusion, the study shows for the first time that combining Olive Leaf extract with Metformin can significantly reduce viability of MCF-7 through a strong synergistic inhibitory effect.

Keywords: *Olea europaea*, anticancer activity, drug combination, MCF-7, metformin, MTT, olive leaves extract

Introduction
Cancer is a leading cause of death globally. It is expected that the number of new cases and death by cancer will grow rapidly as the world populations grow, age and adopt lifestyle behaviors that increase cancer risk [1]. The problem of breast cancer is noticeably growing in both hemispheres of the world. According to GLOBOCAN, as of the year 2018, the disease is second on the list of causes of death worldwide, and is responsible for an estimated 9.6 million deaths in the same year [2].

Even though existing conventional anticancer drugs played a major role in treatment of the disease for many years, they still have many drawbacks. More recently, new treatments with new directions have developed by researchers to fight the growing impact of cancer on populations. Among those are hormone therapy, immunotherapy, targeted therapies and angiogenesis inhibitors. Other treatment strategies such as drug repurposing and drug combinations also hold a promising role for therapeutic purposes [3].

Herbal extracts have been used for treatment of ailments for a long time ago and even until now in many impoverished countries. Many pharmaceutical agents have been discovered by screening bioactive compounds from plants. These products have shown potent biological effects, including anticancer activities. Olive tree is a famous crop in the Mediterranean Basin region known for its oil and leaves that contain numerous bioactive compounds. The leaves usually are byproducts of olive fruit processing with no specific usage, but with great availability. Investigating the health benefits of those bioactive compounds have shown some of these properties such as antimicrobial, antioxidant, antitumor and hypoglycemic, and positive effects on the cardiovascular system [4-7]. More recently, studies have shown greater content and medical potential of polyphenol in olive leaves than olive oil. Polyphenols, in general, have many advantages toward the treatment of cancer including: its low toxicity, abundancy, and specificity and broad biological responses. In fact, the most important advantage of these compounds is their differentiation in response between normal cells and cancerous ones [8].

Metformin, a well-known drug for the treatment of type 2 diabetes, is one of the most efficient

metabolic modulators, as well as holding antitumor properties among other impacts. Epidemiological studies have shown decreased risk of cancer among diabetic patients using MET compared with other drugs [9]. The attention has also been given to its ability to inhibit proliferation in different human cancer cell lines. In fact, the drug showed significant synergy when combined with different drugs both *in vitro* and *in vivo* models through wide action mechanisms [10].

Combining drugs can have great efficacy as a strategy for cancer therapy. This efficacy could be decreasing toxicity or lowering doses of drugs, or minimizing or delaying emergence of drug resistance. In fact, combining drugs with different mechanisms or modes of action can be more effective against single target or treating a disease. In this work, the efficacy of combining olive leaves extracts with the generic glucose-lowering drug MET against MCF-7 was investigated.

Material and Methods

Preparation of olive leaves extract

Olive leaves samples, from a cultivar known as "Souri", were directly collected from a sunny farm north of Gaza City, in the middle of May 2018. Olive leaves were first washed thoroughly with deionized water and then dried in an air oven (Boxun, China) for 3 consecutive days at 37°C. Dried leaves were then pulverized in a blender and the obtained powder was kept in a sealed jar in the dark at room temperature until further extraction. 15 g of leaves powder were placed in the thimble of a Soxhlet and extracted using 300 ml 80% Methanol for 6-8 hours (organic solvent extract) or, 300 ml with deionized water for 6-8 hours (aqueous extract). Then, the samples were filtered and evaporated until dryness by Vacuum Centrifugal Concentrator (IR Concentrator, N-Biotek, Korea).

Stocks preparation

Metformin (1, 1-Dimethylbiguanide hydrochloride, Sigma Aldrich LTD) was dissolved in distilled water to make a 300 mM stock solution and then filter-sterilized and stored at-20 °C until further use. Stocks of leaves crude extracts were prepared by dissolving 400mg of the extract with 1ml of DMSO (Sigma) for organic solvent extract. For aqueous extract, water was used as a solvent to make 400 mg stock. All stocks were filter-sterilized and kept at-20 °C until use.

Cell culture and drug treatment

Maintaining cells

MCF-7 cells, kindly provided by Prof. Rana Abu-Dahab (University of Jordan), were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biological Industries) supplemented with 10% Fetal Bovine Serum (Biological Industries), 10 mg/ml penicillin and 10 mg/ml streptomycin (Biological Industries) and incubated in a humidified atmosphere in an incubator with 5% CO_2 at 37 °C. Medium was changed every 3 days and cells were subcultured upon reaching 85% confluency. For subculture, cells in flasks were detached using trypsin/EDTA (Biological Industries) and counted by means of trypan blue (Biological Industries) using a hemocytometer.

Seeding 96-well plates

$1X10^4$ cells/well were inoculated onto 96-wells plate (100µL per well) which was used for 24-hour treatments. For 48-hour incubation treatments plates were inoculated with 8,000 cells/well. For background absorption, some wells were left cell-free and used as blank control.

Treatments

For single treatments, cells were treated with (75, 150, 300, 600, 900 and 1200µg/ml) [11-13] 80% Methanolic and distilled water olive leaves crude extracts. On the other hand, treatment using MET were in concentrations of (5, 10, 20, 30, 40 and 50 mM), both treatments were kept for 24 or 48 hours before MTT assay. Wells seeded with cells but left untreated were used as a negative control. The 50% (IC50) inhibitory concentration was then calculated.

To test the synergistic anti-proliferative effect different ratios (1:4, 1:7, 1:9, and 1:10) of MET combined with OLE (DW) or OLE (Methanol 80%) were used to treat MCF-7 cells in 96-wells plates for 24 hours or 48 hours followed by MTT assay. The combination index (CI) and Dose Reduction Index (DRI) were used to analyze the synergistic inhibitory effects of the combinations.

In vitro cell proliferation (MTT) assay

As an indicator for viability, the metabolic activity of living cells was determined using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After 24 or 48 hours of treatments for both single and combination studies, wells with treated cells were washed with phosphate buffer saline (PBS) (Biological Industries), immersed with 100µl of fresh medium containing 10% MTT and incubated for further 3 hours. All previous work was done in the dark. The medium was removed and intracellular formazan crystals were dissolved in 100µl of DMSO. After 30 minutes, the absorbance was measured at 550 nm using microplate reader (Multiskan FC Microplate Photometer, Thermo Scientific).

Drug combination analysis

According to Chou Talalay method, the combination index (CI) is, "based on concentration effect curves generated as a plot of the fraction of unaffected cells versus drug concentration using the following CI equation: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + (D)_1(D)_2/(Dx)_1(Dx)_2$, where $(D)_1$ and $(D)_2$ are the concentrations of [MET and OLE] that exhibit a determined effect when applied simultaneously to the cells, and $(Dx)_1$ and $(Dx)_2$ are the concentrations of the same drugs that exhibit the same determined effect when used singly. The CI values indicate a synergistic effect when <1, an antagonistic effect when >1, and an additive effect when equals to 1".

qPCR analyses

MCF-7 cells ($7.8X10^5$) were incubated in 25 cm^2 flasks for 24-hours. Each flask was treated as follow: 50 mM Metformin, 300 µg/ml of OLE, and at the IC50 of the combination of both drugs. A flask was left untreated as a control. After 24 hours following treatment, media were removed and cells were harvested using Trypsin EDTA solution. The harvested cells were then washed with PBS and centrifuged at 1500 rpm for 5 minutes. Total RNA was isolated using ISOLATE II Biofluids RNA Kit (BIOLINE, USA) according to the manufacturer's instructions. 500ng of total RNA were converted to cDNA with anchored oligo dT primers and random hexamers using the SensiFast cDNA Synthesis Kit (BIOLINE, USA). PCR amplification of cDNA (1µl per 20µl of PCR reaction) was carried out using a real-time PCR machine (7500 Real-Time PCR System, Applied Biosystems™) using SensiFAST SYMR Lo-ROX kit

(BIOLINE, USA) according to this procedure: 2 minutes at 95 ℃ for Polymerase activation and denaturation of cDNA, followed by 40 cycles, each consisted of 5 seconds at 95 ℃ for denaturation and 30 seconds for primer annealing and chain extension at 59 ℃, 61 ℃, 62 ℃, 60 ℃ and 60 ℃ for CASP8, BAD, TP53, BAX, and TTP, respectively. GAPDH was used as a reference. Primers used are shown in Table 1. Expression data were normalized to the geometric mean of the housekeeping gene GAPDH to control the variability in expression levels and then were analyzed using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001).

Table 1: The forward and reverse primers used for amplification of different genes.

Gene	Primers	Product size (bp)	Gene	Primers	Product size (bp)
CASP8	F: CTGGGAAGGATCGACGATTA	123	TTP	F: CTGTCACCCTCTGCCTTCTC	156
	R: CATGTCCTGCATTTTGATGG;			R: TCCCAGGGACTGTACAGAGG	
BAD	F: CCCAGAGTTTGAGCCGAGTG	249	BAX	F: TGGAGCTGCAGAGGATGATTG	95
	R: CCCATCCCTTCGTCGTCCT			R: GAAGTTGCCGTCAGAAAACATG	
p53	F: TTGCCGTCCCAAGCAATGGATGA	199	GAPDH	F: GGCATTGCTCTCAATGACAA	199
	R: TCTGGGAAGGGACAGAAGATGAC			R: TGTGAGGGAGATGCTCAGTG	

Hoechst 33342 staining

6-wells cell culture plates were seeded with $2.4X10^5$ MCF-7 cells per well then incubated overnight to adhere. Cells were treated with concentrations corresponding to single and combinations treatments of OLE and MET. After appropriate incubation, sufficient Hoechst 33342 staining solution (5 µg/ml) covered the cells to stain DNA, and incubated for 10 minutes in the dark. Morphological changes were detected using a fluorescence microscope (AMG: EVOS, Thermofisher USA).

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were separated from whole heparinized blood of a healthy donor by centrifugation through a density gradient using Leucoprep™ (iNtRON Biotechnology) Lymphocyte Separation Medium. Cells were counted using Trypan Blue and then resuspended in appropriate nutrient medium.

Data analysis

All of the experiments were performed in triplicates. All acquired data were analyzed using Microsoft Excel; and IC50 and Combination Index (CI) calculations were done using CompuSyn (ComboSyn Inc.). Tables and graphs were produced and edited using GraphPad Prism 6.

Results

Impact of OLE on the MCF-7 cells proliferation

Treatments of MCF-7 cells were performed with various doses of OLE for 24 and 48 hours. Decrease in cell viability of MCF-7 cells in dose- and time-dependent manners were observed, as assessed by the MTT assay. The inhibitory effect on MCF-7 cells increased from 23 to 82% using OLE (DW) and from 11 to 88% using OLE (Methanol 80%), as the concentration of both extracts increased from 75 to 1200 µg/ml (Fig 1A-1B). Furthermore, the antiproliferative activity of OLE was found to be stronger after 48 hours, as concluded from the IC50 values. The IC50 value of OLE (DW) was found to be 300 and 182 µg/ml for 24 and 48 hours, respectively. The IC50 values of OLE (Methanol 80%) was found to be 322 and 135 µg/mL for 24- and 48-hours, respectively.

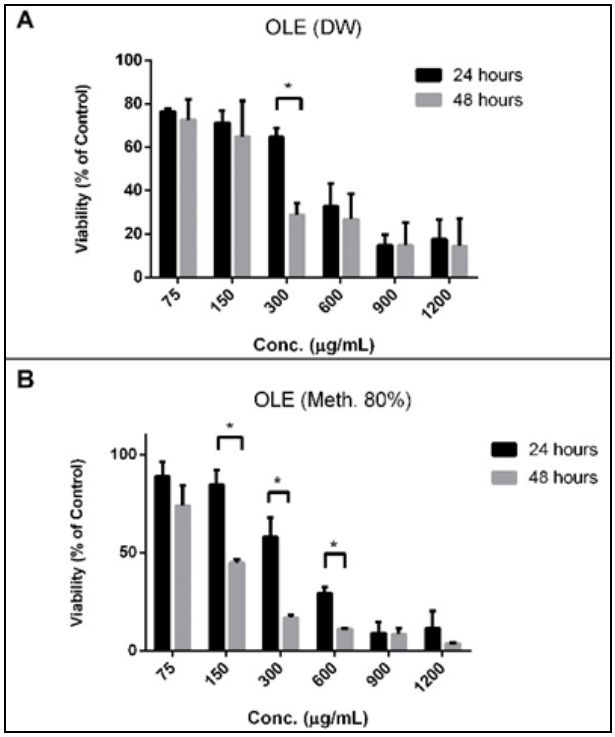

Fig 1: A: Effect of OLE extracted using DW on MCF-7 at 24 and 48 hours; **B:** Effect of OLE extracted using Methanol 80% on MCF-7 at 24 and 48 hours. Values are expressed as mean ± SD of at least 3 independent experiments. *, $P < 0.05$.

Impact of metformin on the MCF-7 cells proliferation

MCF-7 cells were exposed to various doses of MET for 24- and 48-hours. The MTT assay revealed a decrease in the MCF-7 cells viability in dose- and time-dependent manner. As the concentration of MET increased from 5 to 50 mM, the inhibitory effect on MCF-7 cells increased from 6 to 34% and 8 to 79% for 24- and 48-hours incubation, respectively (Fig 2). On the other hand, the antiproliferative activity of MET was found to be stronger on the 48-hour treatment. The IC50 of MET was found to be 78 mM and 21 mM for 24- and 48-hours treatments, respectively.

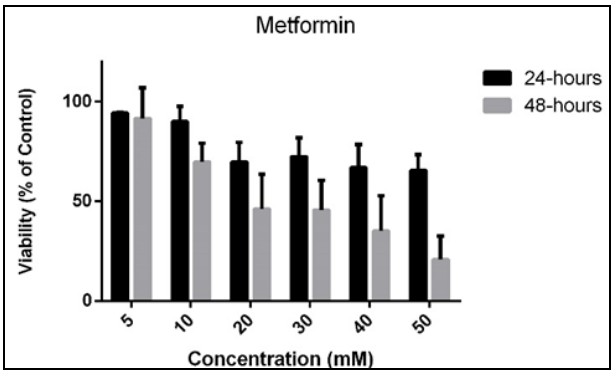

Fig 2: Effect of different single concentrations of MET on MCF-7 at 24 and 48 hours. Values are expressed as mean ±SD of at least 3 independent experiments.

Combination studies

Combined effect of metformin and olive leaves methanolic extract on the MCF-7 Cells

The combined effect of MET and OLEs (Methanol 80%) on the proliferation of MCF-7 cells were assessed after 24- or 48-hours treatment. Combined treatments were performed using various ratios. After 24-hour incubation, at 0.5 fraction and 1:7 MET/OLE ratio, the combined (IC50) dose was reduced to 166. The dose of MET was reduced by 3.7 folds and that of OLE by 2.2 folds. The CI showed synergistic effect at all fractions (Table 2). At 1:4 ratio, the combined dose (MET/OLE) was 120, at 0.5 fraction. The dose of MET was reduced by 3.2 folds and for the OLE by 3.4 folds. The CI of each fraction increased in synergy as concentrations of both drugs increased (Table 3).

Table 2: Inhibitory effect of combining metformin with OLE (Meth. 80%) on MCF-7 at 24 hours with ratio (1:7).

	Single Dose		Metformin combined with OLE (Meth. 80%) 24-hour (1:7 ratio)				
Fa	MET.	OLE	CI	Combined Dose	MET. & OLE	DRI	
						MET.	OLE
0.25	25	168	0.71	71	10+61	2.9	2.7
0.5	78	322	0.71	166	21+145	3.7	2.2
0.75	237	618	0.76	392	49+343	4.8	1.8
0.9	720	1184	0.83	920	115+805	6.2	1.4

The CI values indicate a synergistic effect when CI <1, an antagonistic effect when CI >1, and an additive effect when CI is equals to 1; Fa: Fraction affected; DRI: Dose Reduction Index. OLE: Olive Leaf Extract; MET: Metformin; CI: Combination Index.

Table 3: Inhibitory effect of combining Metformin with OLE (Meth. 80%) on MCF-7 at 24 hours with ratio (1:4).

	Single Dose		Metformin combined with OLE (Meth. 80%) 24-hour (1:4 ratio)				
Fa	MET.	OLE	CI	Combined Dose	MET. & OLE	DRI	
						MET.	OLE
0.25	25	168	0.99	79	16+63	1.6	2.6
0.5	78	322	0.60	120	24+96	3.2	3.4
0.75	237	618	0.39	181	36+145	6.6	4.3
0.9	720	1184	0.26	274	55+219	13	5.4

The CI values indicate a synergistic effect when CI < 1, an antagonistic effect when CI >1, and an additive effect when CI is equals to 1; Fa: Fraction affects; DRI: Dose Reduction Index; OLE: Olive Leaf Extract; MET: Metformin; CI: Combination Index.

For the 48-hour treatments, and at 1:7 ratio, the MET-OLE combination reduced the IC50 dose of MET by 3 folds and that of OLE by 2.7 folds (Table 4). The CIs of all fractions showed synergistic inhibitory effect (Fig 3A).

Table 4: Effects of combining Metformin with OLE (Meth. 80%) at 48 hours with ratio (1:7).

	Single Dose		Metformin combined with OLE (Meth. 80%) 48-hour (1:7 ratio)				
Fa	MET.	OLE	CI	Combined Dose	MET. & OLE	DRI	
						MET.	OLE
0.25	10	62	0.89	34	4+30	2.4	2.0
0.5	21	135	0.69	56	7+49	3.0	2.7
0.75	43	290	0.54	91	11+80	3.8	3.6
0.9	87	625	0.42	149	19+130	4.7	4.8

The CI values indicate a synergistic effect when CI <1, an antagonistic effect when CI >1, and an additive effect when CI is equals to 1; Fa: Fraction affects; DRI: Dose Reduction Index; OLE: Olive Leaf Extract; MET: Metformin; CI: Combination Index.

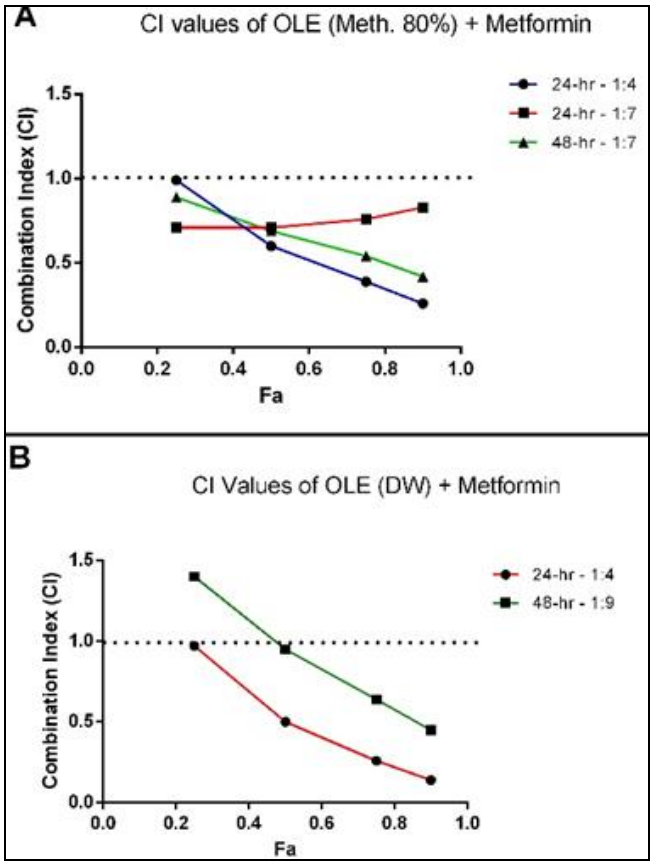

Fig 3: A: The CI values of different combination ratios of OLE (Meth. 80%) to Metformin against different Fa values; **B:** The CI values of different combination ratios of OLE (DW) to Metformin against different Fa values. The CI values indicate a synergistic effect when CI <1, an antagonistic effect when CI > 1, and an additive effect when CI is equals to 1; Fa: Fraction affects; CI: Combination Index

Combined effect of metformin and aqueous extract of olive leaves on the MCF-7 Cells

The combined effect of MET and OLEs (DW) on the proliferation of MCF-7 cells were assessed as described above for the methanolic OLE. The CI values of different MET-OLE (DW) combinations at different Fa's are shown in (Fig 3B). After 24 hours incubation, at ratio of 1:4, the combination showed synergistic inhibitory effect and the combined IC50 dose was reduced to 97. The MET dose was

reduced 4.0 folds and that of OLE 3.9 folds. Most Fa's showed synergistic effects (Table 5). After 48 hours incubation an additive effect was observed at 1:9 ratio **(**Table 6).

Table 5: Effect of combining Metformin with OLE (DW) at 24 hours with ratio (1:4).

	Single Dose		Metformin combined with OLE (DW) 24-hours (1:4 ratio)				
Fa	MET.	OLE	CI	Combined Dose	MET. & OLE	DRI	
						MET.	OLE
0.25	25	113	0.97	65	13+52	1.9	2.1
0.5	78	300	0.5	97	19+78	4.0	3.9
0.75	237	793	0.26	145	29+116	8.1	6.8
0.9	720	2097	0.14	215	43+172	16	12

The CI values indicate a synergistic effect when CI< 1, an antagonistic effect when CI > 1, and an additive effect when CI is equals to 1; Fa: Fraction affects; DRI: Dose Reduction Index; OLE: Olive Leaf Extract; MET: Metformin; CI: Combination Index.

Table 6: Effects of combining metformin with OLE (DW) at 48 hours with ratio (1:9).

	Single Dose		Metformin combined with OLE (DW) 48-hours (1:9 ratio)				
Fa	MET.	OLE	CI	Combined Dose	MET. & OLE	DRI	
						MET.	OLE
0.25	10	62	1.4	60	6+54	1.7	1.1
0.5	21	182	0.95	98	10+88	2.1	2.1
0.75	36	420	0.64	160	16+144	2.6	3.7
0.9	87	1586	0.45	262	26+236	3.3	6.7

The CI values indicate a synergistic effect when CI<1, an antagonistic effect when CI >1, and an additive effect when CI is equals to 1; Fa: Fraction affects; DRI: Dose Reduction Index; OLE: Olive Leaf Extract; MET: Metformin; CI: Combination Index.

Morphological changes of MCF-7 cells

Membrane blebbing

Plasma membrane protrusions (blebs), as followed under the inverted microscope, were observed upon treating MCF-7 cells with MET and OLE (Figure 4). Those could be due to apoptotic death of the treated cells.

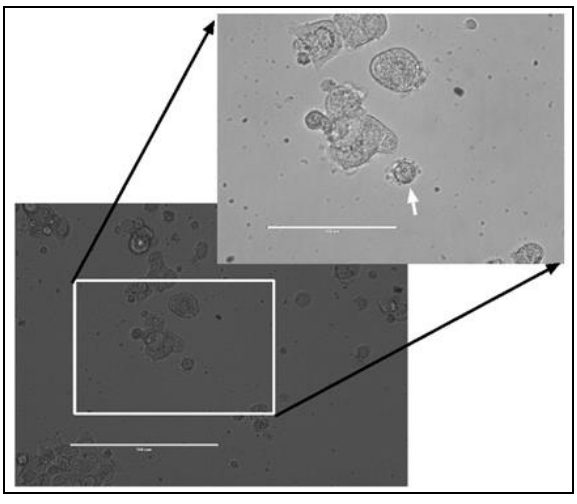

Fig 4: Morphological changes of MCF-7 treated with 50 mM of MET and 200µgmL-1 of OLE (DW) for 48-hr. Membrane blebbing is indicated by a white arrow as observed using inverted Microscope under zooming by 40X.

Chromatin condensation study

Upon cell staining with Hoechst 33342, untreated control cells looked homogenous in shape and color (Fig 5A) however, treated cells either were reduced in number, and chromatin condensation resulted in smaller and brighter color nuclei; (Fig 5B-5D).

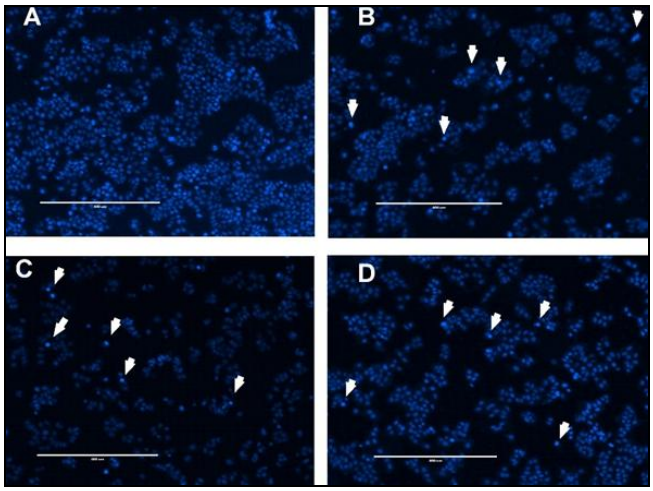

Fig 5: Effect of MET and/or OLE (DW) on the nuclear condensation of MCF-7 cells after 48 h. MCF-7 cells were treated with either MET or OLE or in combination for 48 hours, stained using Hoechst 33342 dye and then observed under fluorescence microscope with 10X magnification. Chromatin condensation indicated by White arrows. A: Untreated MCF-7 cells (Control); B: Cells treated with 60 µg mL-1 of OLE (DW); C: Cells treated with 20 mM of MET; D: Cells treated with 7 mM MET and 49 µg OLE (DW) in combination.

Apoptotic and autophagy marker genes' expression

In order to investigate whether specific apoptosis markers may be implicated in the inhibitory effects of MET and/or OLE (DW) on MCF-7; *TP53*, *BAD*, *BAX* and *CASP8* genes expressions were investigated using qPCR and compared to untreated controls. Results are shown in (Fig 6). The expression of *TP53* was reduced in the three treatments as compared to control; however, *CASP8* and *BAD* expressions were increased in MET-only treatment; and not in the OLE or the combination treatment. Moreover, the expression of the zinc finger protein, Tristetraprolin (TTP), was also increased in MET-only treatment.

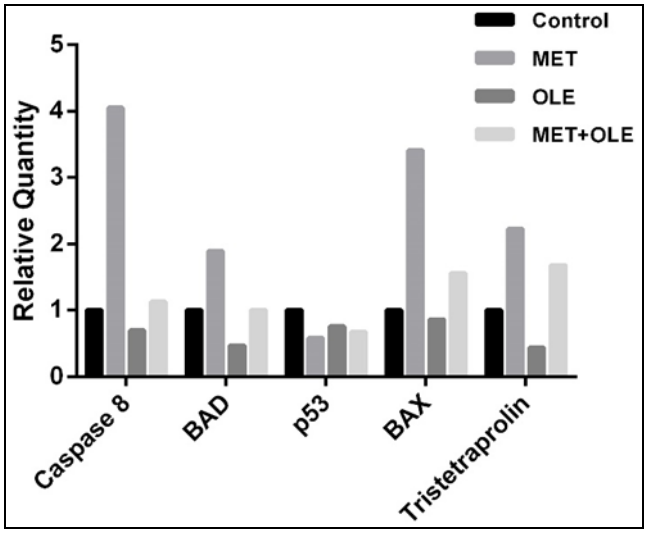

Fig 6: Relative expression, using qPCR, of some Apoptotic markers. Cells were treated with single or combination doses of Metformin and OLE (DW) for 24 hours on MCF-7 cell line. MET: Metformin; OLE: Olive Leaf Extract.

Impact of OLE or/and metformin on normal PBMCs

Cytotoxic effects of both MET and OLE, singly or in combination, were tested *in vitro* on PBMC's. Those two drugs did not show any significant cytotoxicity towards healthy human PBMC's as compared to MCF-7 tumor cells (Fig 7).

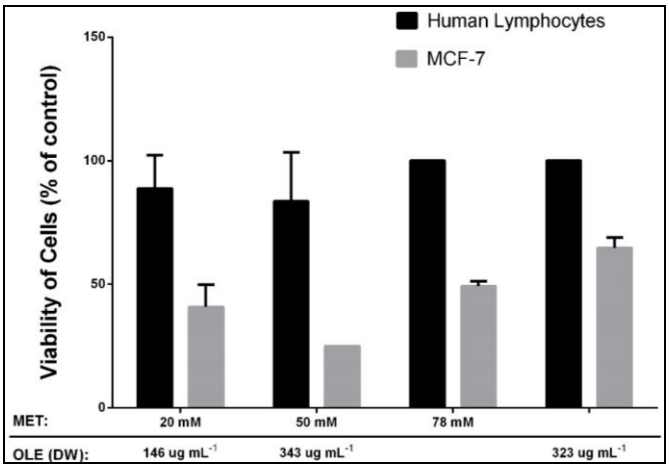

Fig 7: Viability of both MCF-7 and Normal Human Lymphocyte after single and Combined treatments of both MET and/or Olive Leaf Extract (DW), all concentration were made at experimental IC50, except for (50 mM + 343ug mL-1 combination) which is IC75; all after 24-hours; OLE: Olive Leaf Extract; MET: Metformin

Discussion

Alternative cancer treatment strategies are highly needed. Plant-derived bioactive compounds, as they are natural and readily available, may be one important candidate for this purpose. In addition, repurposing of existing drugs e.g., metformin, with potential anticancer activity can be also beneficial in treating cancer as this strategy saves time and money for the health systems. Many reported studies, including epidemiological and *in vitro* ones, have shown that MET and OLE, singly, possess anticancer activity on different cancer cell lines including MCF-7 [9, 14-17]. Therefore, in this study we investigated the effect of MET and OLE administered in combination on MCF-7 cancer cell line; aiming at achieving beneficial synergistic or additive effect.

Antiproliferative activity of metformin and OLE in single doses on MCF-7

In a study by Goulas *et al* (2009), two solvents were used in extracting Olive leaves, Methanol and distilled water and agitation was used for extraction. The IC50 after 48-hour treatment was 209 µg/mL for water extract, and 174 µg mL^{-1} for methanolic extract on MCF-7 [18]. On the same line, our study showed that the IC50 of water and methanol OLE were 182 µg mL^{-1} and 135 µg mL^{-1}, respectively. In another study by Bouallagui *et al* (2011), the results showed that treatment of MCF-7 cells with 3000 µg mL^{-1} of 80% Methanolic OLE showed 38% inhibition [15]. Our results showed far greater potency in inhibition at this fraction. In our study and in general, OLE using 80% Methanol in single dosage has shown stronger inhibitory effect on MCF-7 cells than OLE using water. These results were consistent with most previous studies. On the other hand, reason for variations between our study and other studies maybe attributed to extraction methods and bioactive compound contents due to geographical locations where samples were obtained.

Many studies have confirmed our results for antiproliferative property of MET on MCF-7. In a study by Zhang *et al* (2017), at concentration 20 mM MET, viability of MCF-7 was 40%; on the other hand, in our study the same results were obtained, viability of MCF-7 at the same concentration was 46% [19]. In another study, Queiroz *et al* (2014) also yielded same viability percentage in which 68% viability resulted by treating MCF-7 cells with 10 mM MET, whereas, in our study 69% viability resulted by treatment with same concentration [16].

Antiproliferative activity of combining Metformin/OLE on MCF-7

Only few studies tested the anticancer effects of the crude extract of olive leaves combined with other drugs. In this study, we combined OLE with MET, for the first time, in different ratios according to their IC50's that were deduced from single OLE and MET treatments.

Generally speaking, combining MET with OLE, at particular ratios, has dramatically decreased cell proliferation as compared to single treatments. In fact, the highest effective outcome was 90% inhibition of cell viability. The 1:4 ratio of MET and OLE (DW) showed "Strong Synergism" with a CI=0.14 after 24-hour treatment. In this particular combination, dose reduction index for both MET and OLE (DW) were 16 and 12-folds, respectively. Furthermore, the second most synergistic effect (CI=0.39) was at 75% inhibitory effect and at ratio 1:4 after 24-hour treatment. In addition, dose reduction index at this combination of MET and OLE (Meth. 80%) was 13 and 5.4-folds, respectively.

In a recent study Falah *et al*. (2017) used MET in combination with the natural product curcumin against various breast cancer cell lines. Their results showed additive to moderate synergistic effect. For MCF-7 following 48-hour treatment they observed additive effect (CI=1.069) at IC50=22.5 mM and 108µM of MET and curcumin, respectively [20]. Whereas in the present study, at IC50, combining MET with OLE (Meth. 80%) has shown synergism (CI=0.69) at 48-hour treatment, reflecting a more potent inhibitory effect.

In another study conducted by Liu *et al*. (2012), the authors investigated the effects of combining MET with Carboplatin and other chemotherapeutic drugs. At 50% inhibitory effect, the combination index for MET with carboplatin was 0.814 which indicates a "Moderate Synergism". In the same study, similar results were achieved using doxorubicin or paclitaxel, both are breast cancer chemotherapeutics [21]. In this study, most of our CI's at this fraction have shown more potent synergism. In addition, in the current study we didn't use a chemotherapeutic drug, instead, we used a relatively safer natural extract; adding more advantage to our combination.

As a complex disease, multiple targets in cancer cells are needed to be taken into consideration when treating the disease. Single targets have shown drawbacks for treatments; however, multitargeting by multiple compounds, which is found in natural crude extracts, can work simultaneously to provide a significant effect. Still, the network of pathways and the specific bioactive compounds that cause the effects of combination need to be considered in follow-up research.

Potential mechanism of action of metformin and OLE

The present study results showed that treatments containing MET singly or combined with OLE elicited some signs (e.g., membrane blebbing, chromatin condensation, and increased expression of certain apoptotic genes, including *BAD*, *BAX* and *CASP8*) indicative for apoptosis induction. In agreement with our results Malki and Youssef (2011) have shown that MET induced apoptosis in MCF-7 cells [17]. Additionally,

Zhang *et al*. (2017) have shown that treatment of several cell lines, including MCF-7 with MET for 48-hour showed early and late apoptotic features in a dose-dependent manner [19]. Unexpectedly, our results showed that p53 expression wasn't upregulated upon MET or (MET+OLE) treatment. Meanwhile, the upregulation of TTP indicates that the apoptosis observed here occurred through a TTP-mediated p53-independent pathway. The work of Pandiri *et al*. (2016), Baou *et al*. (2009) and Gondi *et al*. (2007) showed that the anti-proliferative effect of MET is mediated via TTP in a p53-independent manner. [22-24]. Additionally, Falah *et al*. (2017) have shown that MET-induced apoptosis occurred by a p53-independent manner [20].

OLE seems to elicit its antiproliferative effect in a different way. The work of Bouallagui *et al*. (2011) and others have shown that OLE effects its antiproliferative action through cell cycle arrest [15]. Thus, decreasing the cell number and resulting in an apparent cytotoxic effect revealed with MTT. OLE either alone or combined with M*Et al*so seems to downregulate the expression of the apoptotic genes investigated in this study. Similar findings were reported by Elgebalya *et al*. (2018) who showed that olive oil and leaf extract decrease the expression of apoptosis markers [25]. The different cytotoxic mechanisms of OLE and MET may also explain the strong synergism attained in the combination treatments experiments.

The use of purified constituents of OLE, olive leaves collected from different cultivars, and obtained from trees grown in different soils can help elucidate the appropriate cytotoxic mechanism of OLE extract.

Cytotoxicity of both metformin and OLE on normal cells

The safety of normal human cells by any therapeutic drug must be considered before any further research. In fact, safety is one of the main issues of current conventional therapies for cancer. OLE has shown great discriminatory property between normal and cancerous cells which makes them a potential candidate for cancer treatment. In addition, MET has been in the market for many years without any major safety issues. In this study, single and combination treatments of OLE and/or MET have been tested for their antiproliferative effect on PBMC's. Both single and combination treatments had minimal effect on PBMC's; thus, indicating that MET, OLE, and their combination might be safe for human use.

References

1. Torre LA, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends-An Update. Cancer Epidemiol Biomarkers Prev. 2016; 25(1):16-27.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68(6):394-424.
3. National Institutes of Health, Understanding Cancer. Bethesda (MD), 2007.
4. Qabaha K, Al-Rimawi F, Qasem A, Naser SA. Oleuropein Is Responsible for the Major Anti-Inflammatory Effects of Olive Leaf Extract. J Med Food. 2018; 21(3):302-305.
5. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontology a Biol Sci Med Sci. 2014; 69(1):S4-9.
6. Wainstein J, Ganz T, Boaz M, Bar Dayan Y, Dolev E, Kerem Z *et al*. Olive leaf extract as a hypoglycemic agent in both human diabetic subjects and in rats. J Med Food. 2012; 15(7):605-10.
7. Perrinjaquet-Moccetti T, Busjahn A, Schmidlin C, Schmidt A, Bradl B, Aydogan C. Food supplementation with an olive (*Olea europaea* L.) leaf extract reduces blood pressure in borderline hypertensive monozygotic twins. Phytother Res. 2008; 22(9):1239-42.
8. Ramos S. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. Mol Nutr Food Res. 2008; 52(5):507-26.
9. Heckman-Stoddard BM, Decensi A, Sahasrabuddhe VV, Ford LG. Repurposing metformin for the prevention of cancer and cancer recurrence. Diabetologia. 2017; 60(9):1639-1647.
10. Iliopoulos D, Hirsch HA, Struhl K. Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. Cancer Res. 2011; 71(9):3196-201.
11. Samet I, Han J, Jlaiel L, Sayadi S, Isoda H. Olive (*Olea europaea*) leaf extract induces apoptosis and monocyte/macrophage differentiation in human chronic myelogenous leukemia K562 cells: insight into the underlying mechanism. OXID Med Cell Longev. 2014; 2014:927619.
12. Goldsmith CD, Vuong QV, Sadeqzadeh E, Stathopoulos CE, Roach PD, and Scarlett CJ. Phytochemical properties and anti-proliferative activity of Olea europaea L. leaf extracts against pancreatic cancer cells. Molecules. 2015; 20(7):12992-3004.
13. Mijatovic SA, Timotijevic GS, Miljkovic DM, Radovic JM, Maksimovic-Ivanic DD, Dekanski DP *et al*. Multiple antimelanoma potential of dry olive leaf extract. Int J Cancer. 2011; 128(8):1955-65.
14. Han J, Talorete TP, Yamada P, Isoda H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Cytotechnology. 2009; 59(1):45-53.
15. Bouallagui Z, Han J, Isoda H, Sayadi S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. Food Chem Toxicol. 2011; 49(1):179-84.
16. Queiroz EA, Puukila S, Eichler R, Sampaio SC, Forsyth HL, Lees SJ, *et al*. Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PLoS One. 2014; 9(5):e98207.
17. Malki A, Youssef A. Antidiabetic drug metformin induces apoptosis in human MCF breast cancer via targeting ERK signaling. Oncol Res. 2011; 19(6):275-85.
18. Goulas V, Exarchou V, Troganis AN, Psomiadou E, Fotsis T, Briasoulis E, *et al*. Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. Mol Nutr Food Res. 2009; 53(5):600-8.
19. Zhang J, Li G, Chen Y, Fang L, Guan C, Bai F, *et al*. Metformin Inhibits Tumorigenesis and Tumor Growth of Breast Cancer Cells by Upregulating miR-200c but Downregulating AKT2 Expression. J Cancer. 2017; 8(10):1849-1864.
20. Falah RR, Talib WH, Shbailat SJ. Combination of metformin and curcumin targets breast cancer in mice by angiogenesis inhibition, immune system modulation and induction of p53 independent apoptosis. Ther Adv Med Oncol. 2017; 9(4):235-252.
21. Liu H, Scholz C, Zang C, Schefe JH, Habbel P, Regierer AC *et al*. Metformin and the mTOR inhibitor everolimus (RAD001) sensitize breast cancer cells to the cytotoxic

effect of chemotherapeutic drugs *in vitro.* Anticancer Res. 2012; 32(5):1627-37.
22. Pandiri I, Chen Y, Joe Y, Kim HJ, Park J, Chung HT *et al*. Tristetraprolin mediates the anti-proliferative effects of metformin in breast cancer cells. Breast Cancer Res Treat. 2016; 156(1):57-64.
23. Baou M, Jewell A, Murphy JJ. TIS11 family proteins and their roles in posttranscriptional gene regulation. J Biomed Biotechnol. 2009; 2009:634520.
24. Gondi CS, Kandhukuri N, Dinh DH, Gujrati M, Rao JS. Down-regulation of uPAR and uPA activates caspase-mediated apoptosis and inhibits the PI3K/AKT pathway. Int J Oncol. 2007; 31(1):19-27.
25. Elgebaly HA, Mosa NM, Allach M, El-Massry KF, El-Ghorab AH, Al Hroob AM *et al*. Olive oil and leaf extract prevent fluoxetine-induced hepatotoxicity by attenuating oxidative stress, inflammation and apoptosis. Biomed Pharmacother. 2018; 98:446-453.