Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

Mitigating Efficacy of *Inula Graveolens* (L.) Desf. (Asteraceae) In Breast Adenocarcinoma MCF7 and T47D Proliferation: *In Vitro* Mechanistic Studies of A Selected Ethnomedicinal Plant from Jordan

Received for publication July, 6, 2016
Accepted, January, 14, 2017

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ABSTRACT

Inula graveolens (L.) Desf. and I. viscosa (L.) Aiton (Asteraceae) are traditional phytomedicine plants from Jordan. ***Materials and Methods****: Both Inula species were evaluated for their antiproliferative activity against MCF7 and T47D breast cancer cell lines by the sulforhodamine B assay.* ***Results:*** *Unlike inactive I. viscosa but comparable to the marked antineoplastic efficacies of cisplatin and doxorubicin; I. graveolens ethanol extracts were substantially active with respective IC_{50} values (µg/mL) of (3.8 ± 0.3) and (10.9 ± 1.3) against MCF7 and T47D cells. The I. graveolens- apoptogenic mechanism was further investigated by determining the levels of p53, p21/WAF1, FasL (Fas ligand), and sFas (Fas/APO-1). I. graveolens induced a highly pronounced augmentation of MCF7-caspase-8 ($p<0.001$ vs. untreated basal wells). Nevertheless I. graveolens proapoptotic efficacy was not specifically p53/p21- or sFas/FasL- dependent and lacked significant enrichment in cytoplasmic mono- and oligonucleosomes in relation to DNA fragmentation ($p>0.05$ vs. untreated basal wells). The strong antiproliferative activity of its phytoprinciples quercetin (IC_{50} values (µg/mL)) (16.9±4.2) and (23.7±2.5) and luteolin (5.3±0.4) and (4.3±0.1) against MCF7 and T47D cells, respectively, justifies the plant's anticancer use in the traditional medicine.* ***Conclusions:*** *I. graveolens proapoptotic-antiproliferative extracts may be useful in breast cancer mitigation/management regimens.*

Keywords: Breast Cancer; *Inula* spp., Apoptosis, MCF7, T47D, Jordan

1. Introduction

Breast cancer is now the most prevalent cancer in developed and developing countries despite improved preventive and detection measures [1-3]. Apoptosis is governed by a complex network of effector molecules. It is characterized by DNA fragmentation, cell shrinkage and nuclear condensation, and phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane bilayer [4]. Furthermore, targeting the extrinsic apoptosis signaling pathway for cancer therapy may incur modulation of multiple targets as well as phytopharmaceutical-synergism in overcoming multidrug resistance [5-6]. *Inula graveolens* (L.) Desf. (syn. *Dittrichia graveolens* (L.) Greuter) is a poisonous annual plant of Mediterranean origin [7]. It is commonly known as a stinkwort within the sunflower family. It has been comprehensively profiled phytochemically. A sesquiterpene lactone, graveolide, along with several sesquiterpenes, lactones and benzoic acid derivatives, sesquiterpene acids, flavonone, dihydroflavonols, flavones and aromatics were isolated [8-9]. Along its reputed antimycobacterial and veterinary phytotherapeutic roles [10-11]; bactericidal effect was reported for *I. graveolens* essential oil and its volatile constituents could exert appreciable anti-*Candida albicans* effect *in vitro* [12-13]. Additionally anticholinesterase activity was outlined for *I. graveolens* commercial essential oil and its volatile components [14]. Allegedly antiinflammtory

and antipyretic as well as antimicrobial properties also were ascribed for its eudesmane derivatives and sesquiterpenes [15]. Its significant and selective phytotoxic activity was recently recognized of its constitutive 2, 3, 11β, 13-tetrahydroaromaticin and ilicic acid [16]. Therefore in continuation of our interest in the antiproliferative activity of aromatic medicinal plants used in the traditional medicine in Jordan, and to establish the cancer chemotherapy/prevention action mechanism of *I. graveolens*, **the present study was designed to** assay the levels of p53, p21, Fas ligand and sFas and caspase -8 activity, which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents [17-20].

2. Materials and Methods

2.1. Chemicals and kits

Unless stated otherwise, all reagents and chemicals were procured from Sigma (Dorset, UK). Cisplatin and doxorubicin were procured from local suppliers. The Quantum Protein assay kit (EuroClone, Siziano, Italy), nucleosome ELISA (Roche Diagnostics, Mannheim, Germany), ELISA kits for human total p53, total p21, FasL and sFas, the caspase-8 colorimetric assay kit (all from R & D Systems Europe, Abingdon, UK) were used.

2.2. Plant material

Flowering aerial parts of *I. graveolens* and *I. viscosa* were collected from Al-Jubeiha region, in the vicinity of the University of Jordan, Amman (31°57'N and 35°56'E), Jordan, during late summer 2012. The plants were identified by Prof. Barakat Abu Irmaileh (Department of Plant Protection, Faculty of Agriculture, The University of Jordan). The leaves were air dried at room temperature (RT) in the shade until constant weight, and subsequently assayed for essential oil composition. The respective voucher specimens (AST21/FMJ and AST11/201, respectively) have been deposited in the Department Pharmaceutical Sciences/ Faculty of Pharmacy/ The University of Jordan, Amman-Jordan.

2.3. Extracts preparation for anti-proliferative assay

Plant flowering aerial parts were powdered coarsely. Ten grams were weighed and gently boiled with 100 ml of the solvent for 10 minutes. Four different solvents were used; water, ethanol, chloroform and ethylacetate. Preparations were covered and left overnight. The next day filtration was followed by evaporation until dryness. Each 0.1 g plant extract was dissolved in 10 mL DMSO. To 15 µL of this preparation fresh media (RPMI 1640 or DMEM/F12) was added to complete volume up to 3 mL for initial screening of the biological activity of the plant extract. For those extracts that showed potential activity, IC_{50} values were determined by preparing appropriate dilutions in the corresponding growth media.

2.4. Determination of the antiproliferative/cytotoxic activity

The cell lines under investigation were human breast adenocarcinoma cell line fully detailed in **Table 1**. Human periodontal fibroblasts (PDL), which are a primary cell culture, were kindly provided by Dr. Suhad Al-Jundi and Dr. Nizar Mhaidat from The Jordan University of Science and Technology, Irbid, Jordan. The 7th-15th passage of PDLs was used in these experiments. All media were fortified with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin. Cells were seeded in 96 well-plates and after 24 h incubation, 100 µL of the extract was added. Initially crude extracts, fractions and phytoprinciples as well as antineoplastic drugs were initially dissolved in dimethylsulfoxide and

then diluted with the growth medium and passed through a 0.2 µm filter. The solution was diluted with growth media and serial dilutions were made (0.1 - 200 µg/mL) before the addition of 100 µL to the cells. Incubation followed for 72 h and afterwards cell viability was determined using sulphorhodamine B assay (SRB) [21]. As positive controls, cisplatin and doxorubicin were procured and their respective IC_{50} values were repeatedly calculated.

2.5. Assessment of apoptosis by ELISA

Apoptosis of MCF7 cells was assayed using the nucleosome ELISA kit, which monitors cytoplasmic histone associated DNA fragments. MCF7 cells were incubated for 72 h with vehicle alone (1% DMSO, control) and with the extracts at the respective IC_{50} values.

2.6. Assays of apoptosis-related proteins

ELISA assays of *p53, p21/WAF1, FasL* and *sFas (Fas/APO-1)* were performed according to the manufacturer's protocols. MCF7 cells were treated as above, and cell lysates were prepared using the respective kit-specific lysis buffer. Subsequently, the lysates were placed in 96-well plates coated with monoclonal antibodies against sFas, p53, p21, or FasL, respectively, and incubated for 2 h at RT. After removing the unbound material by washing buffer, a second incubation with sandwich antibodies followed. Thereafter, horseradish peroxidase-conjugated streptavidin was added and peroxidase activity determined by measuring the absorbance at 450 nm. Thereafter, concentrations of p53, p21, FasL, and sFas were directly determined by interpolating from standard curves. Results are presented as the percentage of the change relative to the untreated control.

2.7. Assay for caspase-8 activity

As per the manufacturer's instructions, cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37^{o} C. The release of *p*-nitroaniline was monitored at 405 nm. Results are presented as the percentage of the change of the activity relative to the untreated control.

2.8. Statistical analysis

Results were expressed as means (as % of control) ± S.D. (standard deviation). Statistical comparisons of the results were made by ANOVA followed by Dunnett's post-test whenever appropriate using Graphpad Prism (version 3.02 for windows; GraphPad Software, San Diego, CA, USA). Values of the means of untreated control and treated cells were considered significantly different if $P < 0.05$.

3. Results

3.1. Cell viability

Table 2 illustrates the distinctive antiproliferative activities of the reference antineoplastic drugs cisplatin and doxorubicin by IC_{50} values mainly characterized for both breast adenocarcinoma cell lines MCF7 and T47D. *I. viscosa* crude extract (50 µg/mL), nevertheless, could poorly mitigate cell viability by respective %proliferation reduction of 25.0 ± 2.1 % and 20.0 ± 1.7 % along with comparable lack of PDL fibroblast cytotoxicity. Reportedly where maximum reduction in cell viability was seen after 72 h of incubation; the corresponding IC_{50} values (µg/mL) of the ethanol extracts of *I. graveolens* were (3.8 ± 0.3) and (11.0 ± 1.3) in MCF7 and T47D cell incubations. Moreover the antiproliferative effects of its chloroform, butanol and ethylacetate fractions are tabulated along with the plant's bioactive phytoprinciples

quercetin and luteolin (**Table 2 and Figure 1**). In the respective plant treatments of ZR751, Vero and PDL Fibroblasts wells, *I. graveolens* impressive antiproliferative IC_{50} values (µg/mL) were (25.0±4.5), (7.0±0.5) and (47.0± 2.6) respectively.

3.2. Effects of *Inula graveolens* on induction of apoptosis as detected and quantified by nucleosome ELISA

A quantitative evaluation of apoptosis was sought using nucleosome ELISA to detect the amount of DNA fragmentation. Compared with basal (non induced) control wells, *Inula graveolens* IC_{50} value of 3.8 ± 0.3 µg/mL did not induce a significantly detectable enrichment in the cytoplasmic mono- and oligonucleosomes after assumed induction of programmed MCF-7 cell death ($p > 0.05$, 113.0 ± 11.4% vs. control (non-induced) wells 101.0± 14.2%; **Figure 2**).

3.3. Effect of *Inula graveolens* on receptor-mediated apoptosis-related molecules

Previous reports have indicated that MCF-7 cells have a normal (non-mutated) tumor suppression gene, p53. In examining the effects of *I. graveolens* on cell cycle regulatory molecules, including p53 and its downstream molecule p21, **Figure 3** demonstrates that *I. graveolens* IC_{50} value of 3.8 ± 0.3 µg/mL lacked any augmentation of the expression of either protein at the examined incubation time ($p > 0.05$, 99.5 ± 6.1% vs. control (non-induced) wells 100.0 ± 6.3% and 108.0 ± 10.2% vs. 100.0 ± 6.7% basal control wells). Thus, *I. graveolens* pro-apoptotic efficacy might not be regulated by either p53 or p21. Additionally, to further establish the sequence of events involved in *I. graveolens* induction of apoptosis, the recruitment of sFas/FasL-mediated execution of apoptosis was investigated. Two major distinct apoptotic pathways have been described for mammalian cells. Fas receptor-mediated apoptotic signaling is one of the most important extrinsic apoptotic pathways in cell demise. Binding of Fas to oligomerized Fas ligand (FasL) activates apoptotic signaling through the death domain that interacts with signaling adaptors including Fas-associated protein with death domain (FADD) to activate caspase-8 which unites in the effector caspase-3 to mediate the rapid dismantling of cellular organelles and architecture. Over 72 h, sFas/FasL system was not also recruited in *I. graveolens*-mediated inhibition of proliferation in MCF-7 treatment ($p > 0.05$; 94.0 ± 11.1% vs. control (non-induced) wells 99.0 ± 5.4% and 105.0 ± 2.6% vs. 99.0 ± 5.7%; **Figure 4**). We next examined the downstream caspase of sFas/FasL system, as the hallmarks of the apoptotic mechanism include the activation of cysteine proteases, which represent both initiators and executioners of cell death., surprisingly, *I. graveolens* IC_{50} value of 3.8 ± 0.3 µg/mL increased caspase 8 activity selectively and highly significantly (152.0 ± 3.9%, n=4, $p<0.001$ vs. basal (non induced) control wells 102.0 ± 2.8%, **Figure 5**), inconsistent with lack of sFas/FasL system modulation.

4. Discussion

Carcinogenesis is characterized by the partial suppression of apoptosis, which in turn gives tumors a selective advantage for survival and can cause current chemotherapy approaches to be ineffective. Recent progress in understanding the mechanisms of apoptosis has provided potentially new targets for therapy [22]. Apoptosis usually proceeds by two pathways: the intrinsic pathway and the extrinsic pathway [23]. The intrinsic pathway begins with the release of mitochondrial cytochrome c. The extrinsic pathway is mediated by the direct interaction between so-called ‘death ligands’ and ‘death receptors’, in which caspase-8 is activated. When death ligands such as tumor necrosis factor α (TNF α), TNF α-related apoptosis-inducing ligand

(TRAIL) and Fas binds to their receptors, a protein complex called the death inducing signaling complex (DISC) is formed. DISC formation is followed by the activation of caspase-8, which activates caspase-3, in turn executing apoptosis [22].

4.1. *I. viscosa* lack of selective cytotoxicity in breast cancer cell line panel

Inula is a large genus of more than a 100 species within the Asteraseae family. An eight out of a total of sixteen *Inula* species were reported in the literature to have vast ethnopharmacological applications and ideally had been subjected to biological evaluations and/or phytochemical studies [24]. The strong antiproliferative capacity of *I. graveolens* essential oil was basically ascribed to its constitutive caryophylline oxide [25]. *I. viscosa* is a phytochemically closely related species commonly found in the Mediterranean region. Nevertheless it was presently proven inactive against the breast adenocarcinoma cell panel MCF7 and T47D. Among its highly enriched flavonoids and sesquiterpenes content; *I. viscosa* methylated quercetins proved to have outstanding *in vitro* proapoptotic antiproliferative and antimicrobial properties [26-27] with substantial hypoglycemic [28-29]; pronounced abortifacient, anti-implantation and luteolytic effects [30]. *I. viscoa* phytochemical fingerprinting was extensively and comprehensively examined [31-35].

4.2. Augmentation/enhancement of pro-apoptotic mechanisms in breast cancer cell lines are among the new strategies for development of breast cancer management/ mitigation regimens

Drugs that directly induce apoptosis can eliminate cancer cells totally and thus avoid tumor recurrence, unlike drugs resulting in cytostasis which usually leads to tumor relapse. For these reasons, new therapeutic strategies for carcinogenesis should involve targeting of apoptosis regulators to improve response rates, and several such approaches are being investigated *in vitro* in breast cancer cell lines [17, 36-39]. As such, further studies will be needed to explore the potential of approaches that induce cancer cell death by molecular apoptosis agonistic augmentation. The molecular mechanisms during *I. graveolens*-mediated growth inhibition in MCF-7 did not engage either p53 or p21. Also, DNA fragmentation possibility was also not clearly detected. We propose, on the basis of our studies, that *I. graveolens* inhibits cell growth and promotes apoptosis regardless of p53 status. The p53, tumor suppressor protein, is a key cell cycle regulator which responds to DNA damage. Tumors defective in p53 are also considered resistant to apoptosis. Thus targeting cancer cells based on signaling pathways and cell cycle checkpoints is considered to be an effective anticancer therapeutic option that has been shown evidently for some natural chemicals [40-43]. As caspase-8 is the key initiator caspase, it is recruited by the adaptor molecule Fas/APO-1 associated death domain protein to death receptor upon Fas ligand binding. We did not observe any marked alterations of either Fas/APO-1 or Fas ligand levels, nevertheless, caspase-8 was activated highly substantially in treated MCF-7 cells. This may incur speculations about averting the DISC (death-inducing signaling complex) formations in the Fas mediated transduction of activation signals in the extrinsic pathway (receptor mediated cell death) of apoptosis. Interestingly, a considerable cross-talk exists between the extrinsic and intrinsic pathways. Caspase-8 can activate Bid cleavage, which then facilitates cytochrome c release from the mitochondria and subsequent activation of caspase -9 and -3, triggering, thereafter, the mitochondrial dysfunction and disruptions of membrane potential [44]. Similarly, several apoptotic signaling pathways and specific proteins have been described playing a role in apoptosis induced by curcumin [45-46]. This steers future directives

and trends towards more elaborated and in-depth investigations into the biology of *I. graveolens*-induced molecular apoptosis overlapping networks.

4. Conclusions

Succinctly antiproliferative activities of *I. graveolens* ethanolic extracts were described for MCF7 and T47D cell lines and found to be related to extrinsic proapoptosis pathway. Taken together Extracts from *I. graveolens* may be useful in breast cancer management/ mitigation via receptor - mediated pathway but not to the exclusion of other multiple mechanisms.

Acknowledgement This research was funded by a SRTD grant (JO/2008/RGS/034) The Higher Council of Science and Technology. The Deanship of Scientific Research at The University of Jordan is also thanked for financial support.

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Table 1. Cell lines used and their properties/characteristics

Cell Line	ATCC no.	Description	Seeding Density (cell/well)	Growth Media	ER Status	P53 Status
MCF7	HTB-22	Epithelia adenocarcinoma	5000	RPMI 1640	+ (Low Her level)	Wt
T-47D	HTB-133	Ducal carcinoma	10000	DMEM/F12	+ (Moderate Her level)	Mu
ZR-75-1	CRL-1500	Ducal carcinoma	10000	DMEM/F12	+ (Varying Her level)	
BT-474	HTB-20	Ducal carcinoma	10000	DMEM/F12	+ (High Her level)	
Vero	CCL-81	African green monkey kidney	5000	RPMI 1640	-	
PDL	-	Primary periodotal human fibroblasts	10000	RPMI 1640	+	

Table 2. Antiproliferative IC_{50} values of *Inula graveolens* extracts, fractions, phytoprinciples and reference drugs. Results present the average and standard deviation of at least two determinations on two cell passages and each is an average of at least three replicates

Antiproliferative IC_{50} values (μg/mL)	MCF7	T47D
Inula graveolens		
Ethanol extract	3.8 ± 0.3	11.0±1.3
Chloroform extract	19.0 ± 2.0	21.0 ± 2.4
Butanol extract	17 ± 4.4	16.0 ± 4.2
Ethylacetate extract	37 ± 2.4	16± 0.9
Quercetin	17± 4.2	24 ± 2.5
Luteolin	5.3 ± 0.4	4.0± 0.1
Cisplatin	8.0 ± 2.5	11.0 ± 5.3
Doxorobicin	0.1± 0.03	0.3± 0.1

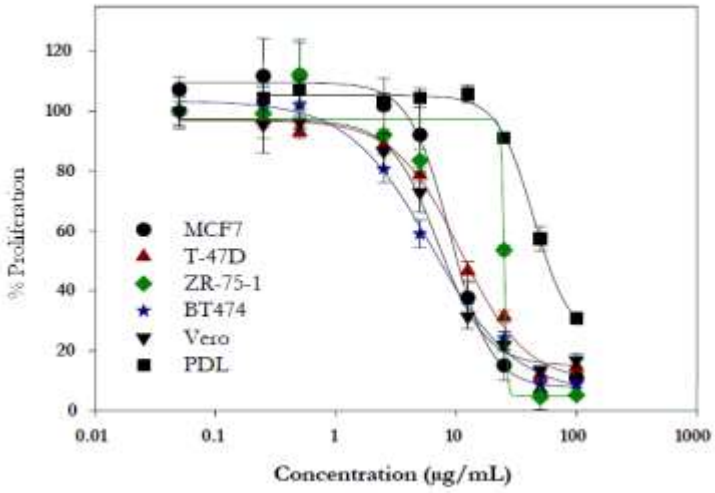

Figure. 1- The Dose dependent antiproliferative effects of *Inula graveolens* crude ethanol extracts against diverse breast adenocarcinoma and fibroblast cells. Results present the average and standard deviation of at 3 replicates.

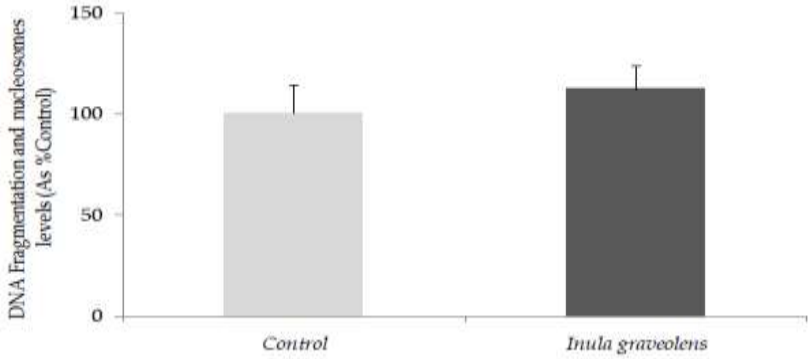

Figure. 2- Effects of antiproliferative *Inula graveolens* extracts on proapotosis DNA fragmentation. MCF7 cell lysates containing cytoplasmic oligonucleosomes of apoptotic cells were analyzed by means of nucleosome ELISA kit. Results expressed as %control are mean ± SD (n=4 independent determinations). None of bioactive plant extracts had a significant statistical difference compared to control (non-induced) wells.

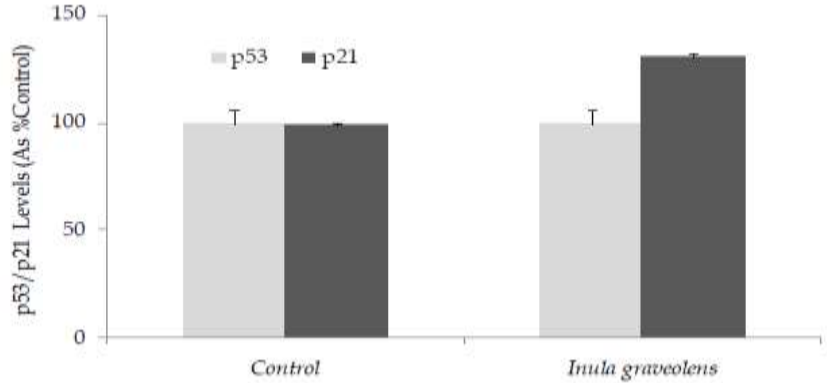

Figure. 3- The lack of effects of antiproliferative *Inula graveolens* on protein expression of p53/p21 in Human breast epithelial adenocarcinoma MCF7 cells, as determined by p53/p21 ELISA kits. Results expressed as %control are mean ± SD (n=4 independent replicates). None of plants' incubations had a significant statistical difference *vs.* control (basal non- induced) wells.

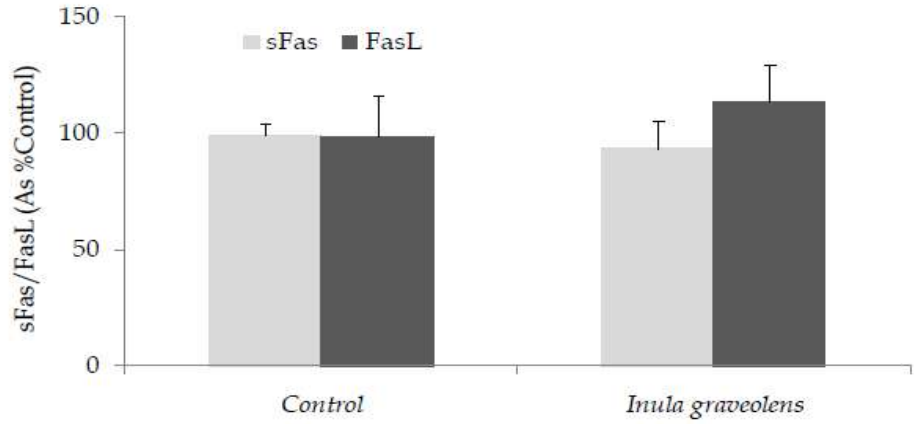

Figure. 4- The lack of Fas/FasL apoptotic system involvement in *Inula graveolens* induction of apoptosis in MCF7 cells. Results expressed as %control are mean ± SD (n=4 independent replicates). None of the bioactive plants'-treatment wells had a statistically significant difference *vs*. control (non-induced) incubations.

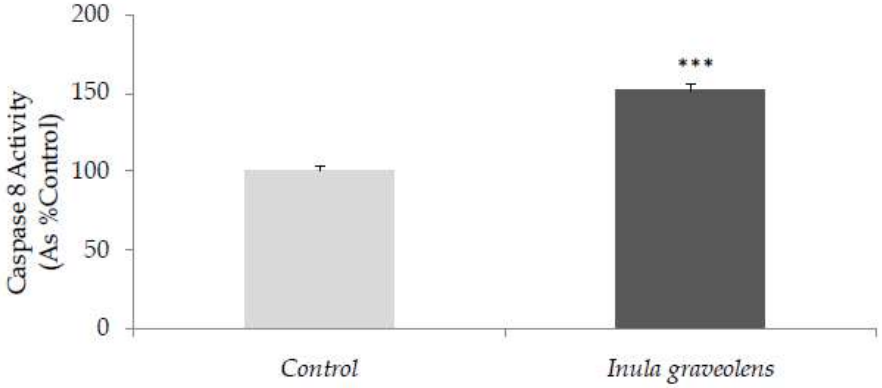

Figure. 5- The activation of caspase -8 in MCF7 cellular model by antiprolifertaive *Inula graveolens* extracts. Results expressed as %control are mean ± SEM (n=4 independent determinations). ***P<0.001 indicates that bioactive plants'-treatment wells had a statistically high significant difference *vs*. control (non-induced) incubations, as analyzed by ANOVA followed by Dunnett's test.