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RESEARCH ARTICLE

EVALUATION OF DIFFERENT EXTRACTION METHODS FROM *PUNICA GRANATUMPEELS* AND ITS ANTI PROLIFERATIVE ACTIVITY AGAINST COLORECTAL CANCER CELL LINE HCT-116

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ABSTRACT

Objective: to investigate the effect of the different types of solvents (water, ethanoland methanol) and extraction methods (ultrasound and maceration) on *P. granatum* peels and their antiproliferative activity against colorectal cancer cell line HCT116.

Methods: The antiproliferative (determined by Neutral red assay) effect of different extraction methods from *Punicagranatum* peels was tested on the epithelial ovarian cancer (SKOV-3) cell line.

Results: The results obtained revealed that the solvents and the extraction methods had a significant effect against HCT116. For the extraction methods applied, the maceration extraction method showed that it has a better anti-proliferative effect compared to the ultrasound method. In addition, the methanol peels extracted by maceration technique showed the highest antiproliferative activity on the HCT-116 cell lines with an IC_{50} of 31.94 ± 4.98 µg/ml.

Conclusion: This study shows than *P.granatum* peels extracts have great potential as future natural antitumor and antioxidant agents.

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INTRODUCTION

The pomegranate, *Punicagranatum,* belongs to the Punicaceae family. This plant is mainly found in Assia, India, China, United State, Mexico and throughout the Mediterranean region. The pomegranate possesses different size, color and taste (Lansky, 2007; Mena, 2013). Pomegranate fruits can be also divided into three parts: the seeds (about 3% of the fruit weight), the juice (about 30% of the fruit weight) and the *peel,* which also include the *interior network of membranes*. Pomegranates were always considered as a sacred fruit that grow in the gardens of Paradise, inHinduism, Persian, Greek, Jewish, Christian, Chineseand in Muslim culture (Seeram, 2006). In many countries and particularly in the Mediterranean region, the pomegranate has been used extensively in the traditional medicine to treat many diseases such as fertility. In the ancient Indian Ayurvedic tradition, the pomegranate was used as an antiparasitic agent and to treat ulcers and diarrhea (Naqvi, 1991; Caceres, 1987).

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In Greco-Arab Medicine, the pomegranate was used to treat diabetes (Zaid, 2013). The pomegranate fruit has been widely used as a traditional remedy against diarrhea, acidosis, microbial infections, helminth infection, snakebite, fever, leprosy, burns, hemorrhage, dysentery and respiratory pathologies (Kim, 2009; Jain, 1984; Siang, 1983; Singh, 1980; Arseculeratne, 1985). Furthermore, the dried pomegranate peels are considered beneficial for the treatment of diarrhea, ulcers, colitis, headache and dysentery (Ismail, 2012). In the Egyptian culture, the dried pomegranate peel was used to treat several disorders such as inflammation, cough, intestinal worms and infertility. The traditional importance of pomegranate as a medicinal plant is now being reinforced by data obtained by modern science. Several studies have demonstrated that the different parts of pomegranate and especially the seeds are rich inpolyphenols (including flavonoids and tannins) which have been proved to be responsible for its antioxidant properties (El-Nemr, 2006).The antioxidant activity of pomegranate juice is at least 20% higher than the other beverages like black apple juice, orange juice, cherry juice, cranberry juice, blueberry juice, grape juice, red wines and iced tea (Seeram, 2008). Pomegranate peel and juice contain high amounts of bioactive compounds especiallyphenolic compounds. The most common phenolic compounds presentincludeflavonoids (anthocyanins and

catechins) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid) (Seeram, 2004). These compounds are responsible for more than 90% of the antioxidant potential of the fruit (Afaq, 2005; Negi, 2003; Zahin, 2010). The ellagitannins including gallic acid and ellagic acid, are responsible for the anticancer, antimutagenic, antidiabetic, anti-inflammatory, antifungal and antimicrobial activity of this fruit (Kasimsetty, 2010; Gil, 2000; Adams, 2010; Yuan, 2012; Adams, 2006; Glazer, 2012; Machado, 2002; Bialonska, 2009). The wide spectrum of health benefits of pomegranate peels have been attributed to its composition of a wide range of phytochemicals, which is why we focused our studyonoptimizing the extraction procedure onthe bioactive compounds in order to obtain the extracts with the best antiproliferative activity. Different types of solvents (water, ethanol and methanol) and extraction methods (maceration and ultrasound) were employed and the obtained extracts were evaluated in terms of in vitro antiproliferative activity againstthe human colorectal cancer HCT-116 cells.

MATERIALS AND METHODS

Fruit collection and powder preparation: Fresh fruits were collected from south Lebanon from an altitude of 300meters. After their collection, fruits were cleaned, washed with water, peeled and the peels were dried in the shade at room temperature and away from sunlight. Dried peels were then grinded to powders and were preserved in clean plastic containers and kept away from light, heat and moisture till use.

Preparation of crude extract by maceration: 15 g of the powdered peels of *P. granatum* were placed in different beakers, each containing150 mL of one of the three different solvents, water, ethanol and methanol. The solution was macerated at room temperature for 24 hrs with continuous agitation. After the preparation of the macerate, the solution was filtered using 0.45 µm filter paper and then concentrated by a rotary evaporator at 40°C with reduced pressure. The obtained extracts were then stored at -20°C to be used in different tests (Zeidan *et al.,* 2014).

Preparation of crude extracts by ultrasound: 1 g of the powdered peels of *P. granatum* dissolved in 50 ml of the different solvents (water, ethanol and methanol) was placed for an hour at 60℃ in the ultrasound apparatus. The obtained extracts were then filtered and evaporated to be stored at - 20 °C (Bandar *et al.,* 2013).

Cells and Cell Culture: Human colorectal cancer cell line HCT-116 was maintained in RPMI-1640 medium (Sigma Chemical Company) supplemented with 0.1mg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (FBS). HCTT-116 was cultured at 37°C with 5% CO_2.

Neutral Red Analysis: Cell viability was performed using Neutral Red assay based on the initial protocol described earlier (Malek*et al.*, 2011; Nasreddine *et al.,* 2015). Neutral Red, a chromogenic dye, is an indicator of a lysosomal activity. Lived cells demonstrated a chromogenic change with Neutral Red which were detected spectrophotometrically. Briefly, cells were detached from the tissue culture flask with 2 ml of trypsin solution. The cell pellet was obtained by centrifugation at 1.000 rpm for 5 minutes. The density of the viable cells was counted by the trypan blue exclusion in a haemocytometer. Cells were then plated in a 96-well microtiter plate, at a concentration of 8×10^3cells/well and incubated ata humidified 37°Cwith 5% CO_2which allows the cells to adhere. After 24 hrs, the cells were treated with five different concentrations of *P. granatum* peel extracts: 50, 100, 150, 200 and 250µg/ml each being tested in triplicates. The plates were incubated for 24, 48 and 72hrs at 37°C with 5% CO_2. The untreated cells were used as a negative control, whilst cells incubated only with buffer (water, ethanol and methanol) were used as a vehicle control. No effect was observed in the buffer. At 24, 48 and 72hrs, the old medium was replaced by100 µl of fresh medium which contains40 µg/ml neutral red and was incubated for 3 hrs. This was done to allow the uptake of the vital dye into the lysosomes of viable and undamaged cells. The media was then discarded and the cells were washed twice with 100 µl of 1X PBS. The intracellular accumulation of neutral red dye was extracted with200 µllysing solution (50% ethanol-1% acetic acid). The optical density (OD) of the eluted dye was measured at 490 nm using a microplate reader. The experiments were conducted in triplicates. The percentage of inhibition of each of the test samples was calculated according to the following formula using the OD values obtained:

Percentage of inhibition (%) = (OD control − OD sample)/OD control × 100.

Statistical analysis: All results were presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with Graph Pad Prism 7 (Graph Pad Software Inc., CA, USA). With Two-way ANOVA test to determine time and treatment effects, respectively. Groups that are significantly different from control are indicated in the figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $P < 0.0001$.

RESULTS

Impact of the different types of solvents and extraction methods of *P.granatum* plantpeelson theviability of HCT-116 cancer cells: An evaluation of the antiproliferative activity of the different types of solvents and extraction methods of *P.granatum* plant peels was done by measuring the viability of the HCT-116 cell line. This evaluation was done using the Neutral Red Cytotoxicity/ Viability Assay after the treatment of the cancerous cell line for 24, 48 and 72 hrs with increasing concentrations (50, 100, 150, 200, and 250 µg/ml) of these extracts. The effect of inhibition by these extracts was dose and time-dependent (Figures 1 and Figure 2). The percentage of inhibition was calculated according to the formula mentioned above. The results of the neutral red assay showedthat the majority of the different types of solvents (water, ethanol, methanol) and extraction methods (ultrasound and maceration) have a significant antiproliferative effect at different doses and different times (Figure 1) while these extracts have no significant antiproliferative effect after 24 hrs of treatment at a concentration of 50 µg/ml (Figure 1 A-F). As shown in Figures 1A & 1B, the concentration of 50 µg/ml of water extract, produced by ultrasound technique during48 hrs and maceration extraction technique during48 and 72 hrs, has no antiproliferative effect. Also, a non significant antiproliferative effect was shown for the water extract produced by the maceration technique as well as a non significant antiproliferative effect was shown for the ethanol extract produced by ultrasound and maceration techniques at the concentration of 100 µg/ml during24 hrs for both. There was also no effect for a concentration of 150 µg/ml during24 hrs for water extract produced by maceration technique (Figure 1 A-E).

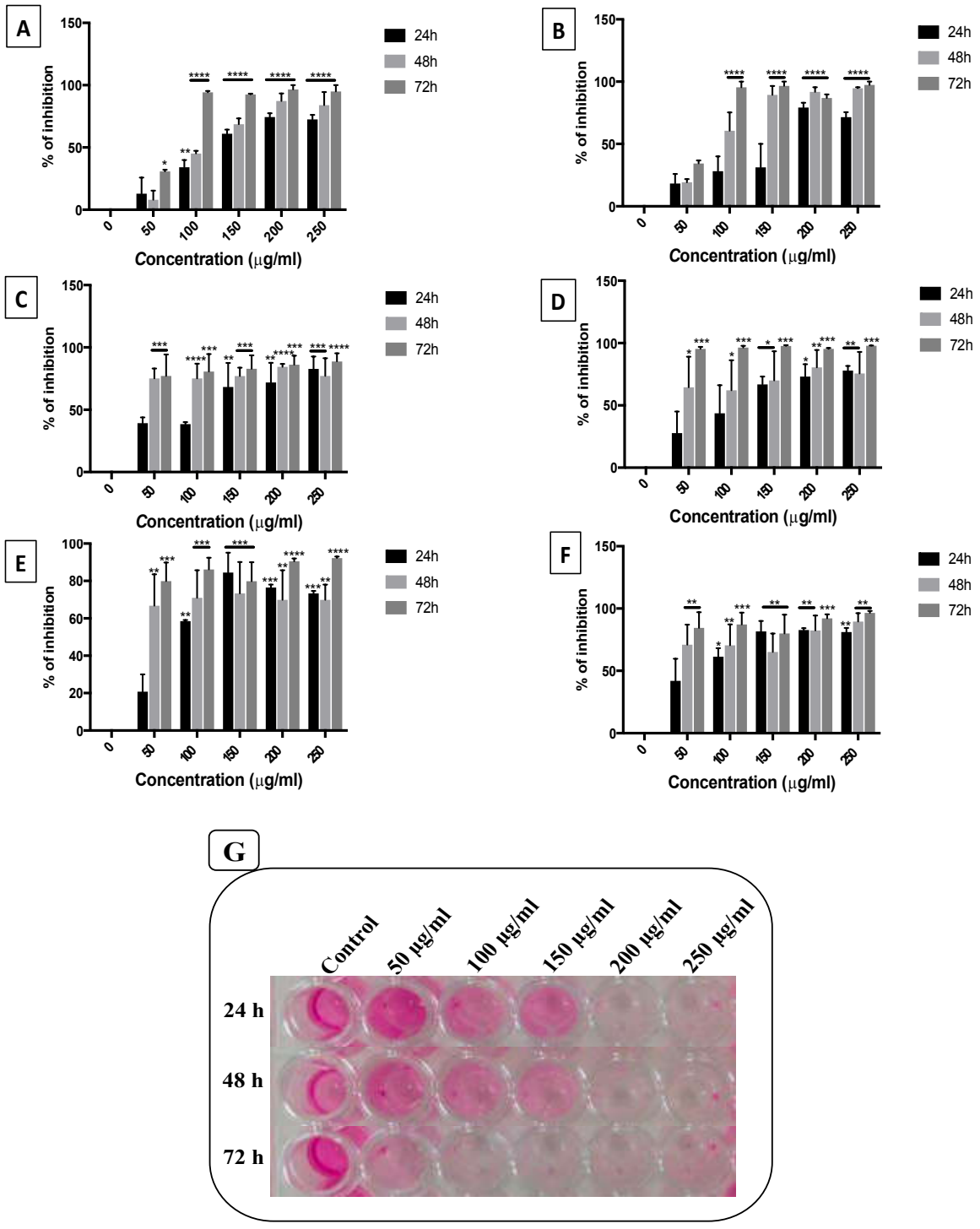

Figure 1.Effects of the different types of solvents and extraction methodson the viability of HCT-116 cancer cellsusing*P.granatum*plant peels. A, F.

The different types of solvents and extraction methods used at a concentration of 250 µg/ml resulted within 72 hrsin ≥ 90 % inhibition ofHCT-116 cells (Figure 1). In addition, the maceration extraction technique, using the different types of solvents, showed an antiproliferative activity greater than that of the ultrasound extraction technique. In order to determine the concentration required to achieve a 50% inhibition of cells induced by each type of solvents and extraction methods, the dose response curve was plotted. The results of the cytotoxic assay are presented as IC_{50} (µg/ml) in Table 1. It was clear from the results obtained that the methanol extract produced by the maceration extraction technique (within24 hrs) has the highest antiproliferative activity on the HCT-116 cell lines with an IC_{50} of 31.94 ± 4.98 µg/ml.

HCT-116 cells were incubated for 24, 48 and 72 hrs with different concentrations (0-250 µg/ml) of different solvents prepared with *P. granatum* plant peels and using different extraction methods. Cell viabilitywasestimated by theneutralred (NR). Percent inhibition of viable cells is calculated through the formula: % Inhibition = OD (opticaldensity) of non-treatedcells - OD of treated cells / OD of non-treated cells × 100. Results represent % inhibtionof cell survivalofHCT-116 cellscompared to control.Each column represents different concentrations of *P.granatum extract* and each line represents different incubation times. (A) Represents the water extract produced by ultrasound extraction technique; (B) represents the water extract produced by maceration extraction technique; (C) represents the ethanol extract

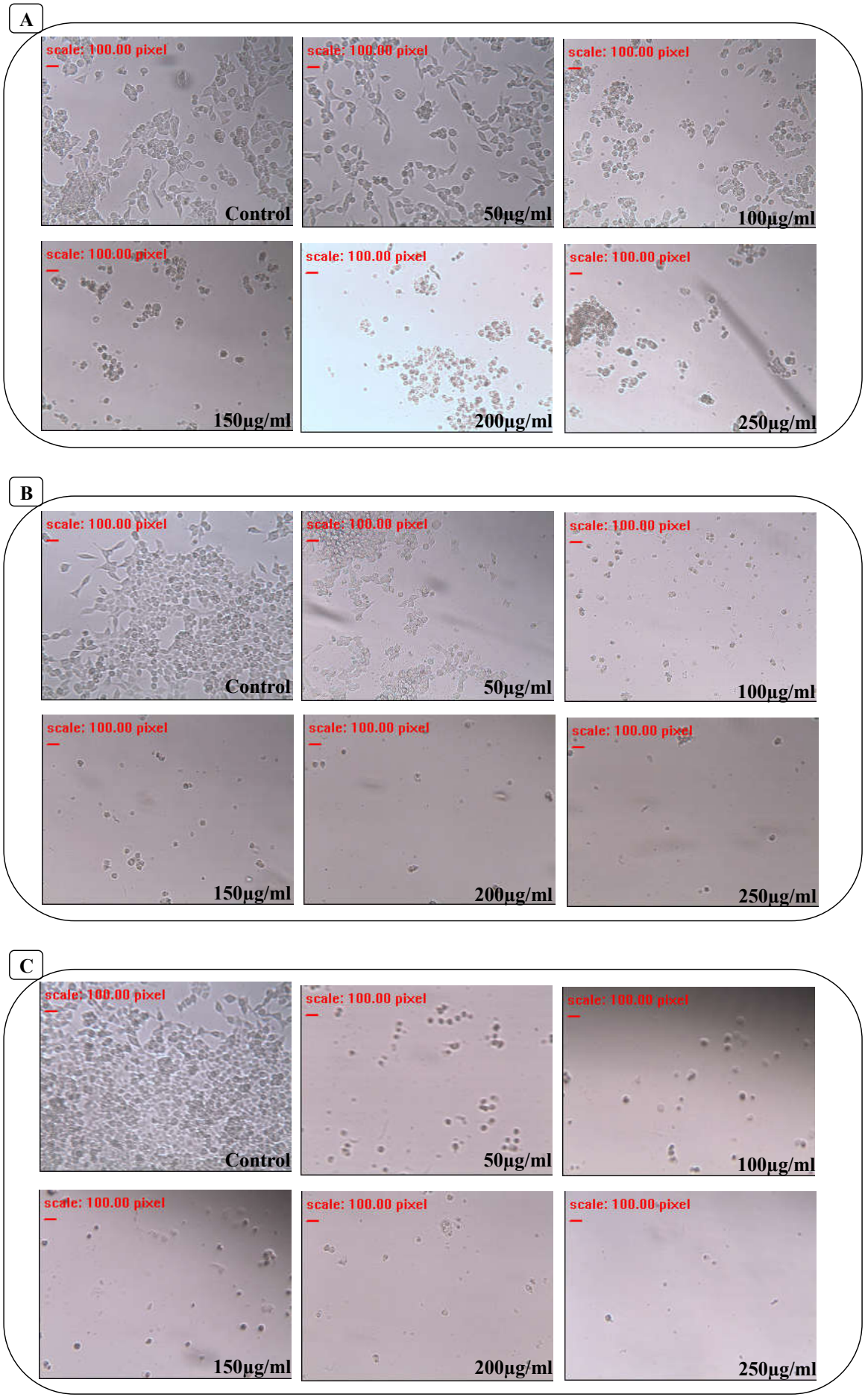

Figure 2. Microscopicview of HCT-116 cellsafter 24 (A), 48 (B) and 72 hours (C) of incubation with increasing concentrations (50, 100, 150, 200 and 250 µg/ml) ofmethanol extract of *P.granatum* produced by maceration extraction technique. The results presented are from one experiment out of three that were carriedout and photographed microscopically (× 40)

Table 1. Concentrations of *P.granatum* extracts produced by different types of solvents and different extraction methods, which induced 50% decrease in HCT-116 cancer cell survival, determined by Neutral red cytotoxicity assay

Type of extracts	Type of techniques	IC_{50} [µg/ml]
Water	Ultrasound	140.1 ± 11.99
Water	Maceration	121 ± 10.83
Ethanol	Ultrasound	78.14 ± 36.38
Ethanol	Maceration	56.5 ± 14.36
Methanol	Ultrasound	84.05 ± 9.08
Methanol	Maceration	31.94 ± 4.98

produced by ultrasound extraction technique; (D) represents the ethanol extract produced by maceration extraction technique; (E) represents the methanol extract produced by ultrasound extraction technique; (F) represents the methanol extract produced by maceration extraction technique. (G) Represents the NR assay plate of stained cells. Each column represents the different concentrations of methanol extract of *P.granatum* produced by maceration extraction technique and each line represents the different incubation times. Experiments were conducted in triplicates and results represent the mean ± SEM (standard error of the mean) of n = 3 independent experiments. The resultant P-value was expressed as * P <0.05; ** P < 0.01; P*** <0.001 was considered to be statistically highly significant and **** P < 0.0001 extremely significant (Two-way ANOVA).

DISCUSSION

In 2015, the World Health Organization (WHO) has identified colorectal cancer as the third leading cause of death globally (774 000 deaths) after lung (1.69 million deaths) and liver (788 000 deaths) cancers. While, in Lebanon, a study conducted by Shamseddine *et al.* (2014), showed that the colorectal cancer ranked as the fourth most prevalent kind of cancer among males and the second among females. Moreover, according to the national cancer registry of the Lebanese Ministry of Public Health (MOPH), the frequency of reported incidents of colorectal cancer increase after the age of 60 in both males and females (Lebanese Ministry of Public Health, 2012). Over the last few years, many studies support the argument that regular consumption of fruits, vegetables, and grains that are rich in polyphenols may reduce the risk of colon cancer (Gossé, 2015; Ramos, 2007; Bobe, 2010). The Pomegranate, which is very rich in polyphenol have also been studied for its protective effects against colon cancer. Seeram *et al.* (Seeram, 2005) reported the effect of pomegranate juice (PJ) and its purified polyphenols on human colon cancer cell lines (HT-29, HCT116, SW480, SW620), and found that PJ displayed the highest antiproliferative and proapoptotic effects compared to its purified polyphenols. So what this study showed that separation of individual polyphenols from PJ may decrease the overall anti-proliferative activity, owing to the requirement of multifactorial effects and chemical synergy of the action of multiple compounds compared to that with the most active single agent alone. Treatment of HT-29 cancer cells with PJ indicates that this juice plays an anti-inflammatory activity in colon cancer cells (Adams, 2006). The anti-inflammatory effect of PJ is also demonstrated in a mouse model, by the down regulation of inflammatory mediators such as COX-2 and iNOS, in colon tissue (Marín, 2013). Several studies suggested that the anticarcinogenic effect of PJ could largely be due to their hydrolysis product ellagic acid, which induced apoptosis in colon cancer cells (Larrosa, 2006). These effects were mediated through the main mechanism of apoptosis, such as introducing cytochrome c in cell cytosol and by up-regulation of pro-apoptotic Bax protein and down-regulation of anti-apoptotic Bcl-2 protein (Larrosa, 2006; Malik, 2005; Seeram, 2004, 2005). In vivo studies have shown the chemo preventive effects of pomegranate and its potential role in colon cancer prevention. The consumption of PJ and pomegranate seed oil (PSO) suppressed the number of aberrant cryptfoci in Fisher 344 male rats with colon *carcinogenesis* induced by *azoxymethane (*Banerjee, 2013; Kohno, 2004). Additional studies based in chemo preventive effects of twenty-six patients with colorectal cancer (CRC) after consumption of pomegranate extract (PE). The study found high level of urolithin in malignant colon tissues after intake of the PE, which exhibit *cancer chemopreventive* activity (Nuñez-Sánchez, 2014). The anti-proliferative effect of pomegranate peel (PP) was studied in different types of cancer, such as prostate (Albrecht, 2004), bladder (Masci, 2016) lung (Zahin, 2014) and breast (Masci, 2016) while its effect in colon cancer has not yet been studied. The aim of this work was to develop the best extraction method for PP in terms to obtain the highest antiproliferative capacity. For the extraction methods applied, the maceration extraction method showed that it has a better antiproliferative effect compared to the ultrasound method. In addition, the methanol extract showed the highest antiproliferative activity, followed by ethanol and water extract, confirming previous results in literature (Shuhau, 2010; Masci, 2016). This study demonstrated that the use of good extraction method is important to give a best antiproliferative activity.

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