Palestine has a rich and prestigious heritage of herbal medicines. To investigate the impact of variable extraction techniques on the cytotoxic effects of medicinal plant extracts, 5 well-known medicinal plants from Palestine were extracted with 90% ethanol, 80% methanol, acetone, coconut water, apple vinegar, grape vinegar or 5% acetic acid. The resulting 35 extracts were screened for cytotoxic activities against three different cancer cell lines (B16F10, MCF-7 and HeLa) using a standard resazurin-based cytotoxicity assay and Nile Blue A as the positive control. Highly variable toxicities and tissue sensitivity were observed, depending upon the solvent used for extraction. The acetone extract of Salvia officinalis L. exhibited the most potent cytotoxicity (IC_{50} = 14 to 36 µg/ml), but very little sensitivity between the three cell lines. More moderate cytotoxicity with improved tissue sensitivity was observed with coconut water extract of S. officinalis L. (IC_{50} = 114 µg/ml) and methanol extract of Teucrium polium L. (IC_{50} = 104 µg/ml). In this study, acetone consistently gave lower extraction yields but higher cytotoxicity, whereas other solvent systems gave much higher extraction yields with lower cytotoxicity. These results demonstrate how the cytotoxicity of plant extracts can be inversely proportional to the yield, and that solvent selection plays an important role in both factors.

Kew words: Plant extract, natural products, anticancer drug, cytotoxicity.

INTRODUCTION

Cancer is one of the most devastating diseases in both developing and developed countries. Due to a global increase in life expectancy, the incidence of cancer and related mortality rates are dramatically increasing. Treatment options are typically expensive and unavailable in developing countries. New and widely available drugs are therefore needed to provide treatment options. Natural products have provided some of the

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most important cancer chemotherapeutics, largely because they provide structurally complicated molecules that are difficult to access in significant quantities by total synthesis (Mukherjee et al., 2001; Raymond, 2004; Efferth, 2009, 2010; Filip et al., 2011; Siu 2011). The extraction of drug candidates from natural product sources requires a proper selection of plant, extraction method and screening method for discovering bioactive molecules.

Palestine has a rich and prestigious heritage of herbal medicines. More than 700 species of medicinal plants are known to exist, and approximately 63 of these are actively used for the preparation of traditional medicines (Ali-Shtayeh et al., 1998; Sawalha et al., 2008; Ali-Shtayeh and Jamous, 2012). The majority of these plants have already been subjected to chemical analyses. Gas chromatography mass spectrometry (GC MS) spectroscopy, high performance liquid chromatography (HPLC) and other methods have revealed that terpenoids and phenolic compounds are the two main families of secondary metabolites present (Hassan et al., 1979; Aron and Kennedy, 2007; Waterman and Lockwood, 2007; El Hadri et al., 2010; Conforti et al., 2012).

Although many efforts have been focused on deciphering the chemical composition and biological effects of these plants, a systematic study of the effects of variable solvents for extract preparation has not been reported. In this study, variable solvents were used to prepare extracts from 5 Palestinian plants (Olea europaea, Vitis vinifera, Ficus carica, Salvia officinalis and Teucrium polium) and screened for cytotoxic activities. These particular plants have been used in traditional medicine for the treatment of various diseases such as inflammation (Surh et al., 2001; Kaileh et al., 2007), hypertension (Suleiman et al., 1988), and diabetes (Table 1) (Baluchnejadmojarad et al., 2005; Orhan et al., 2006; Eidi et al., 2009). Palestinians have used T. polium for abdominal pain, S. officinalis for relief menstrual pain, V. vinifera for weight loss, F. carica for ulcer treatment and O. europaea for destroying urinary and gall stones. Most of the medicinal plants in Palestine are sold in herbal shops, where most patients seeking herbal therapy are elderly (age of > 55 years) who usually suffer from multiple diseases and cannot afford to buy expensive medications.

One of the key steps in natural product processing is the selection of extraction solvent (Taamalli et al., 2012). The most commonly used solvents are water, methanol, ethanol and acetone. Those solvents are used in neat form or as mixtures. In this study, we used apple vinegar, grape vinegar and coconut water as widely-available and inexpensive replacements for pure organic solvents. The non-flammable and non-volatile nature of these solvents also makes their handling safe and environmental friendly for scale-up of production in developing countries (Diaz-Reinoso et al., 2006; Fontana et al., 2009; Yapo, 2009; Min et al., 2011).

**MATERIALS AND METHODS**

**Plant**

The leaves of S. officinalis, O. europaea, F. carica, V. vinifera and T. polium were collected from the Hebron area of Palestine (Coordinates: 31° 32′ 00″N 35° 05′ 42″E) on April, 2012. Plant characterization was conducted by Dr. Rami Arafeh and voucher specimens were deposited in the Biotechnology Research Center at the Palestine Polytechnic University (Table 1). The fresh leaves were separated and cleaned from dust by tissue paper and placed in the shade inside a well-ventilated room until a

### Table 1. List of screened plants, collected part and their uses in Palestinian traditional medicine

| Scientific name | Common name | Family       | Collected part | Preparation            | Traditional Uses                      | Voucher specimen |
|-----------------|-------------|--------------|----------------|------------------------|---------------------------------------|------------------|
| *Ficus carica* L | Fig         | Moraceae     | Fruit and leaf sap | Direct use             | Anti viral (warts treatment)          | 03/04/2012       |
| *Olea europaea* L | Olive       | Oleaceae     | Leaves         | Decoction of leaves    | Reduces hypertension                  | 03/04/2012       |
| *Salvia officinalis* L | Sage       | Lamiaceae    | Leaves and stems | Decoction of leaves and stems | Antispasmodic, antibacterial      | 04/04/2012       |
| *Teucrium polium* L | Felty germander | Lamiaceae    | Leaves and stems | Decoction of leaves and stems | Antispasmodic                 | 04/04/2012       |
| *Vitis vinifera* L | Grape       | Vitaceae     | Liquid sap of the stem | Direct use             | Skin problems, hair loss              | 06/04/2012       |

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constant weight was obtained. Dried leaves were grounded to a fine powder and the powder was stored at 4°C.

Solvents and chemicals

All solvents were of American Chemical Society (ACS) grade and purchased from Merck. Vinegars were purchased from a local grocery store in Hebron city. Coconut water was collected from coconut fruit and stored at 4°C. Nile Blue A was purchased from Fluka.

Preparation of crude extracts

Extracts were prepared by adding the specified solvent (30 ml) to 1 g of dry powdered material in a corning centrifuge tube (50 ml). The mixture was shaken for 24 h at room temperature (23°C), centrifuged, and the supernatant was filtered through cotton. The filtrate was dried under reduced pressure, and stock solutions of 50 mg/ml in dimethyl sulphoxide (DMSO) were prepared at room temperature and stored at -20°C. Extracts prepared with natural solvents (apple vinegar, grapes vinegar, coconut water) were likewise dried and the extraction yields were calculated by subtracting the dry weight of the natural solvent residue from total weight of natural product extract.

Cell lines

Murine metastatic B16F10 melanoma, breast cancer MCF-7, and cervical cancer HeLa cell lines were obtained from American Type Culture Collection, USA (ATCC), cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivation fetal calf serum (FCS), 2 mM L-Glutamine, 100 U/ml of penicillin (Sigma), and 100 µg/ml of streptomycin (Sigma) and incubated in 5% CO₂ at 37°C.

Cytotoxicity assays

"Alamar Blue" resazurin reduction assays were conducted as described (O’Brien et al., 2000). Cell suspended in 100 µl of DMEM were seeded in 96-well plates at a density of 5 x 10⁴ cells per well and incubated for 24 h. All extracts were serially diluted into supplemented media using a separate 96-well plate, applied to the cells, and incubated for 48 h. Following the incubation, 100 µl of fresh media, (containing 10% (v/v) of a 860 µM solution of resazurin in PBS) was added to the cells, and incubated for 2 to 4 h. The fluorescence intensity of the dye was then quantified by a SpectraMax M5 plate reader using excitation at 560 nm. IC₅₀ values were calculated from the fluorescence intensity values, by using an exponential decay curve fit. DMSO was used as a negative control, whereas Nile Blue A (Lin et al., 1991) was used as a positive control.

Statistical analysis

IC₅₀ values are defined as the concentration of the extract where there is a 50% loss of total metabolic activity as compared to untreated controls and are reported as mean ± standard deviation (SD). IC₅₀ values with 95% confidence limits were calculated using GraphPad Prism 3.3 software (GraphPad Software, Inc., San Diego, CA). p Values less than 0.05 were considered to be significant. All experiments have been conducted in duplicate.

RESULTS

Extract yields

Five Palestinian plants were extracted with seven different solvents to yield 35 extracts in total (Table 2). The isolated yields of the extracts were corrected for non-volatile residues present in the natural solvents. The maximum extraction yields ranging between 63 to 91% were consistently obtained when coconut water was used, suggesting the presence of a “green” surfactant effect. Methanol and ethanol extracts gave yields in the range of 12 to 34%, while the acetate acid solution and vinegars gave highly variable yields ranging between 9 to 41%. Acetone extractions consistently gave lowest percentage yields ranging between 4 to 13%, suggesting greater extraction selectivity.

IC₅₀ values in cell cultures

The plant extracts were screened for their cytotoxic activities in three different cancer cell lines using the "Alamar Blue" resazurin reduction assay (O’Brien et al., 2000). This assay reports the combined effects of proliferation and metabolism on total cellular respiration. In general, the least toxic extracts were prepared using the aqueous solvents: 5% acetic acid, natural vinegars and coconut water, while the most toxic extracts were prepared using alcohol or acetone. Little or no cytotoxic effects were exhibited by F. carica or T. polium extracts, irrespective of the type of solvent used for extraction. In contrast, extracts of S. officinalis prepared using organic solvents exhibited exceptionally potent activities with IC₅₀ values ranging between 14 to 64 µg/ml in all three cell lines tested (Table 2). In contrast, acetone and ethanol extracts of O. europaea exhibited good selectivity between the cell cultures, with IC₅₀ values ranging between 43 to 63 µg/ml for MCF-7 cells, and 170 to 510 µg/ml for B16F10 and HeLa cells.

Acetone extracts of all five plants generally exhibited the highest cytotoxicity as compared to the other extraction solvents used (Table 2, Figure 1). Since acetone extracts of S. officinalis exhibited the most potent cytotoxic activities, we characterized the time dependency of its cytotoxicity in MCF-7 cell cultures. As shown in Figure 2, the rapid action of metabolism inhibition indicates that the extract exhibits a cytotoxic,
Table 2. Extraction yields and IC\textsubscript{50} values for 35 different extracts

| Extract # | Plant         | Solvent           | Extraction Yield (%) | MCF-7 IC\textsubscript{50} µg/ml | B16F10 IC\textsubscript{50} µg/ml | HeLa IC\textsubscript{50} µg/ml |
|-----------|---------------|-------------------|----------------------|----------------------------------|-----------------------------------|---------------------------------|
| 1         | Olea europaea | 90% ethanol       | 34                   | 63±18*                           | 321                               | 490                             |
| 2         | Ficus carica  | 80% methanol      | 32                   | 400                              | 190±18*                           | 440                             |
| 3         | acetone       | 10                | 43±13*               | 170±28*                          |                                   | 510                             |
| 4         | Olea europaea | 5% acetic acid    | 26                   | 430                              | >1000                             | >1000                           |
| 5         | apple vinegar | 41                | >1000                | >1000                            | >1000                             | >1000                           |
| 6         | grape vinegar | 28                | 530                  | >1000                            | >1000                             | >1000                           |
| 7         | coconut water | 91                | 860                  | >1000                            | >1000                             | >1000                           |
| 8         | 90% ethanol   | 12                | 440                  | 880                              | >1000                             | >1000                           |
| 9         | acetone       | 4.0               | 400                  | 720                              | 690                               |                                 |
| 10        | 5% acetic acid| 36                | >1000                | >1000                            | >1000                             | >1000                           |
| 11        | Ficus carica  | grape vinegar     | 21                   | >1000                            | >1000                             | >1000                           |
| 12        | coconut water | 63                | >1000                | >1000                            | >1000                             | >1000                           |
| 13        | Vitis vinifera| 90% ethanol       | 25                   | 870                              | 686                               | 610                             |
| 14        | 80% methanol  | 21                | 400                  | 908                              | 620                               |                                 |
| 15        | acetone       | 5.6               | 62±9*                | 137±3*                           | 336                               |                                 |
| 16        | 5% acetic acid| 24                | 950                  | >1000                            | >1000                             | >1000                           |
| 17        | apple vinegar | 25                | >1000                | 993                              | >1000                             | >1000                           |
| 18        | grape vinegar | 11                | >1000                | >1000                            | >1000                             | >1000                           |
| 19        | coconut water | 65                | >1000                | >1000                            | >1000                             | >1000                           |
| 20        | Salvia officinalis | 90% ethanol       | 19                   | 27±11                            | 35±9*                             | 53±8*                           |
| 21        | 80% methanol  | 24                | 34±7*                | 51±2*                            | 64±5*                             |                                 |
| 22        | acetone       | 13                | 16±3*                | 14±2*                            | 36±4*                             |                                 |
| 23        | 5% acetic acid| 24                | 540                  | >1000                            | 820                               |                                 |
| 24        | apple vinegar | 17                | 400                  | 436                              | >1000                             |                                 |
| 25        | grape vinegar | 11                | 390                  | 542                              | >1000                             |                                 |
| 26        | coconut water | 86                | 114±4*               | >1000                            | 845                               |                                 |
| 27        | Nile Blue A   | Positive control  | 3±1*                 | 3±1*                             | 0.8±0.2*                          |                                 |

Cell viability was determined using a resazurin reduction assay. Results are expressed as mean ± S.D (N= 2). *denotes statistically significant of p < 0.05.

rather than cytostatic activity. MCF-7 cells exhibited the highest sensitivity to the plant extracts, with most lower IC\textsubscript{50} values than the other cell lines evaluated (Daoudi et al., 2013). As compared to
HeLa and B16F10 cells, five to 10-fold lower IC_{50} values were observed in MCF-7 cells for diverse extracts including No.: 1, 3, 9, 17, 28, 29, and 30 (Table 2). In contrast, HeLa cells generally exhibited the lowest sensitivity to the plant extracts. The acetone extract of S. officinalis exhibited the most potent activities in HeLa cells with an IC_{50} of 36 µg/ml followed by ethanol and methanol extracts with an IC_{50} value of 53 and 64 µg/ml, respectively. Moderate activities were observed from the acetone extract of T. polium with an IC_{50} of 173 µg/ml, whereas the acetone extract of V. vinifera gave only weak activity with an IC_{50} of 336 µg/ml. The 14 extracts of O. europaea and F. carica were inactive against HeLa cells.

Since acetone extracts of all five plants exhibited the highest cytotoxicity and S. officinalis exhibited the most potent cytotoxic activities, we further evaluated the effect of different acetone extracts of the five plants and the effect of different solvents of S. officinalis to MCF-7 cells at fixed extract concentration (40 µg/ml). The MCF-7 sensitivity to different plants were in the following order: S. officinalis > O. europaea > V. vinifera > F. carica > T. polium and the MCF-7 sensitivity to S. officinalis extracts were in the following order: acetone > 90% ethanol > 80% methanol > coconut water > 5% acetic acid, apple vinegar, grape vinegar (Figure 3).

**Conflict of Interests**

The author(s) have not declared any conflict of interests.

**DISCUSSION**

Most of the currently used anticancer drugs are highly toxic, expensive, and resistance mechanisms pose a significant problem (Lippert et al., 2008; Petrelli and Giordano, 2008; Hait and Hambley, 2009). There is a continuing need to identify new drug candidates that are more effective, widely available and less toxic. Plants extracts are an important source of potentially useful compounds for the development of new anticancer drugs. Here we investigated solvent extraction effects of five Palestinian medicinal plants for cytotoxic activities in three cancer-derived cell lines. Among the 35 extracts tested, a few exhibited potent activities with IC_{50} values of ≤ 100 µg/ml (Table 2).

The acetone, ethanol and methanol extracts from S. officinalis exhibited highest cytotoxicity against all cell lines tested, with acetone extract being the most cytotoxic (Figure 3). S. officinalis is not currently used for anticancer treatments in traditional Palestinian medicine, but the cytotoxicity of S. officinalis has been previously...
reported (Xavier et al., 2009; El Hadri et al., 2010). An essential oil prepared by sub-fractionation of *S. officinalis* by hydrodistillation has previously been tested against cell lines of murine macrophage, colon cancer, and breast cancer cell lines (El Hadri et al., 2010). The reported IC\(_{50}\) values against murine macrophage, colon cancer and MCF-7 cell lines were reported to be 41.9, 77.3, 213.1 µg/ml, respectively.

Our studies demonstrated extracts of *S. officinalis* exhibit potent cytotoxicities that are dose, time, and solvent dependent. The exceptional cytotoxicities of acetone extracts of *S. officinalis* is reproducible even when the extract solution was kept for one week at room temperature. Other extracts like *O. europaea*, in contrast, exhibited diminished activities if the extract was kept at room temperature for few days. To maintain the cytotoxic activities of *O. europaea* extracts, stock solutions must be freshly prepared and stored at -20°C. Oxidation of phenolic compounds from *O. europaea* might be responsible for this loss in activity (Alu'datt et al., 2011; Kontogianni and Gerothanassis, 2012). The stability and reproducibility of *S. officinalis* extracts suggest the involvement of compounds that are resistant to oxidation. The chemical composition of *S. officinalis* has previously been evaluated, sesquiterpenes α-humulene and trans-caryophyllene were found to be major components (Loizzo et al., 2007; El Hadri et al., 2010). The cytotoxic activity of α-humulene against MCF-7 is reported to be 81 µg/ml, whereas trans-caryophyllene was reported to be less cytotoxic (IC\(_{50}\) > 100 µg/ml). This activity is not correlated with the exceptionally high activity of acetone extract reported here; where the combined effects of various compounds with different cellular targets is likely responsible for the high activity.

Natural apple and grapes vinegars and coconut water are natural solvents which could be used for green technologies to replace organic solvents (Chemat et al., 2012). Although high extraction yields were obtained from natural solvents, almost no cytotoxic activities were observed for the extract with unusual exception of coconut water. Coconut water extracts of *S. officinalis* exhibited high activities against MCF-7 cells with an average IC\(_{50}\) of 114 µg/ml and good selectivity as compared to B16F10 and HeLa cells (Figure 4). More study is needed therefore to evaluate the *S. officinalis*-coconut water mixture as a potential chemopreventive agent against breast cancer.

As compared to the vinegars and coconut water, acetone consistently gave lower extraction yields but higher cytotoxicity. These results demonstrate that high extraction yield is not a key factor for achieving high cellular activity. While *in vitro* cytotoxicity can be an initial indicator of *in vivo* antitumor activities, a wide range of phytocompounds are capable of exhibiting nonspecific cytotoxicity. According to American National Cancer Institute (NCI) (Suffness and Pezzuto, 1990) guidelines, an IC\(_{50}\) < 30 µg/ml is considered to be a promising cytotoxicity, therefore plant extracts with significant cytotoxic activity such as extract No. 22, 23, and 24 should be further assessed using animal models.

**Conclusion**

The results of the present study demonstrated that a number of Palestinian medicinal plants have promising anticancer activities in cell cultures. Depending on the extraction solvent used, these plants exhibited moderate...
to highly potent cytotoxic activities. The cytotoxicity of acetone extract of *S. officinalis* L. was highly reproducible, as the potency remained unchanged even when the extract was left in the presence of oxygen for one week at room temperature. Interestingly, coconut water was found to offer a potential alternative to classical organic solvents; it gave consistently highest extraction yields, and in the case of *S. officinalis* L., highly toxic extracts towards MCF-7 cells derived from human breast cancer. To our knowledge coconut water has never been utilized for the purpose of natural product extraction. Taken together, these results demonstrate how the cytotoxicities of plant extracts depend on the solvent used, and that traditional Palestinian medicinal plants can serve as a source for the discovery of new anticancer agents.

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