Anticancer activity of Rosemary (Rosmarinus officinalis L.) and Oats (Avena sativa L.) Extracts, and their antitumor enhancement of 5-Fluorouracil on Colon Cancer Caco-2 Cell Line

Hadil El-burai, Mazen Alzaharna, Rajy Isleem, Mahmoud El-Hindi and Fadel Sharif

Abstract
Colorectal cancer is one of the commonest tumors worldwide, and the third cause of death among all cancers. Over the years, several herbal plants have proved efficacy in modulating this disease. In this study, plant parts of Rosmarinus officinalis, Avena sativa, Punica granatum and Portulaca oleracea were investigated for their possible antitumor effects. The plants were extracted using Soxhlet using different solvent. Using MTT, the viability of Caco-2 cells was assessed after treatments with the crude extracts. The most potent extracts were treated in a combination manner with 5-Fluorouracil against Caco-2 and quantified using Chou-Talalay method. Results show that R. officinalis chloroforamic and A. sativa ethanolic extracts have given the best antiproliferative and cytotoxic effects with IC50 77 and 67 μg/ml, respectively. Combination experiments of those extracts with 5-Fluorouracil have shown various synergies. The two extracts show potentiality for cancer therapy; however, further studies needed to assess their active components and their effects in vivo.

Keywords: Anticancer Activity, MTT assay, Herbal Extracts, Rosmarinus officinalis, Avena sativa, Punica granatum, Portulaca oleracea

Introduction
Cancer remains one of the leading causes of mortality and morbidity globally. An estimated 18.1 million new cancer cases and 9.6 million cancer deaths were therefor the year 2018 worldwide[1]. Annually, over one million new cases of colorectal cancer are reported and around 600000 patients die from the disease. Colorectal cancer treatment essentially consists of surgery, adjuvant chemotherapy, neoadjuvant radiotherapy and targeted therapy. Unfortunately, drugresistance plays a major role for the low survival rates of this cancer group [2]. In fact, nearly half of metastatic Colorectal Cancer patients are resistance to the mainstay therapy, 5-fluorouracil (5-FU), for this disease; thus, alternative methodologies should be foundtto assist treatment. Medicinal plants have been used for their therapeutic values since the beginning of human civilization. Nature has been a rich source of medicines for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of agents in traditional medicine[3]. Plant-based traditional system of medicine continues to play an essential role in health care, with about 80% of the world’s inhabitants relying mainly on traditional medicines for their primary health purposes [4, 5]. Palestine is one of the countries that is famous for many medicinal plants with about 2953 plant species found in this part of the Mediterranean basin, of which over 700 appear in the published ethnombotanical data[6-8]. This study will investigate the anticancer impact of different natural crude extracts of: Rosmarinus officinalis, Avena sativa, Punica granatum and Portulaca oleracea on Caco-2 cancer cells.

2. Materials and Methods
2.1 Plant collection
Plants were collected from different agricultural areas in Gaza. P. oleracea was collected from agricultural areas in the north of Gaza Strip in August, 2016. R. officinalis was collected from agricultural land in central Gaza Strip in January, 2017. P. granatum was purchased from Gaza’s local markets in Gaza Strip in November, 2016, and A. sativa was collected from agricultural land in eastern Gaza Strip in May, 2016.
Plants were identified at the Biology Department at the Islamic University of Gaza. The investigated plants are listed in Table (1).

<table>
<thead>
<tr>
<th>Binomial name</th>
<th>Vernacular</th>
<th>Arabic Name</th>
<th>Part/s Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Rosmarinus officinalis</td>
<td>Rosemary</td>
<td>Ekleel El Jabal</td>
<td>Atrial parts</td>
</tr>
<tr>
<td>2 Punica granatum</td>
<td>Pomegranate</td>
<td>Romman</td>
<td>Peel</td>
</tr>
<tr>
<td>3 Portulaca oleracea</td>
<td>Purslane</td>
<td>Baqleh</td>
<td>Atrial parts</td>
</tr>
<tr>
<td>4 Avena sativa</td>
<td>Oats</td>
<td>Shofan</td>
<td>Seeds</td>
</tr>
</tbody>
</table>

2.2 Preparation of plants extracts
The aerial parts of the two plants (R. officinalis and P. oleracea), the seeds of A. sativa, and the peels of P. granatum were washed thoroughly with tap water, in order to remove soil, dust and any insects that may be present, and dried on clean tissue papers. Then, all plants were air-dried for one day. Plants were then completely dried by using hot oven (Boxun, China) at 37 °C for one week. Dried plants were pulverized using blender mixer into powder and stored in well-sealed sterile containers.

2.3 Extraction of plants material
The ground plants were extracted using Soxhlet extractor apparatus with different solvents (distilled water, ethanol and chloroform). For all plants, ethanolic, chloroformic and distilled water extracts, were prepared as follows: in a Soxhlet extractor apparatus, 20g of the ground plants parts or seeds were weighed and then wrapped in a filter paper then placed in the thimble of the Soxhlet. The weight/solvent volume ratio was 1/10, the system was left at 60-100 °C according to solvent type for 6-8 hours until the extraction process completed. The extracts were then allowed to evaporate in an infrared (IR) concentrator (N-Biotek, Korea) at 37-40 °C (depending on the type of plant and solvent used). Table (2) illustrates the temperature and time needed for drying the extract and heat mode for each solvent. The dried extracts were stored in a refrigerator at 4 °C in a sterile well-sealed tubes until further use [9].

Table 2: Temperature and time needed for drying the extract by IR concentrator as recommended by the manufacturer (Labconco Corporation).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temp (°C)</th>
<th>Time to dry (min.)</th>
<th>Heat mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>40</td>
<td>270</td>
<td>IR/Heat</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>60</td>
<td>IR/Heat</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>39</td>
<td>IR/Heat</td>
</tr>
<tr>
<td>Chloroform</td>
<td>50</td>
<td>49</td>
<td>IR/Heat</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>34</td>
<td>IR/Heat</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>31</td>
<td>IR/Heat</td>
</tr>
<tr>
<td>Water</td>
<td>50</td>
<td>330</td>
<td>IR/Heat</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>180</td>
<td>IR/Heat</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>129</td>
<td>IR</td>
</tr>
</tbody>
</table>

2.4 Preparation of stock solutions
The first stock solutions of ethanolic and chloroformic crude extracts were prepared by dissolving 400 mg of crude extracts in 1ml of dimethyl sulfoxide (DMSO) (Sigma). The stock was vortexed until completely dissolved, filter-sterilized (0.22µm) and then stored at 4 °C. The distilled water stock solutions of the different extracts were prepared by adding 10 mg from different dried crude extract to 1 ml of distilled water. The working solution was prepared by adding 1µl from the stock solution to 1ml of Dulbecco's Modified Eagle Medium (DMEM) (Biological industries) and then the different concentrations were prepared by serial dilution [10].

2.5 Cell line and cell culture
The cell line used in the present study, the colorectal cancer cell line (Caco-2), was kindly provided by Prof. Rana Abu-Dahab (University of Jordan) from American Type Culture Collection (ATCC) and normal lymphocytes where obtained from the Genetic Diagnosis unit at The Islamic University of Gaza. Caco-2 cell line was maintained in DMEM supplemented with 10% fetal bovine serum (Biological industries), 100 units/ml penicillin and 100 µg/ml streptomycin (Biological industries) at 37 °C in a humidified 5% CO₂ incubator (HH.CP.01, China). The medium was changed every 2-3 days for the stock culture.

2.6 Seeding cells for experiment
After counting cells by using a hemocytometer, the cells were then seeded in a 96-wells plate at a density of 10,000 cells/well for Caco-2 cell line and 40,000 cells/well for normal lymphocyte in 100 µl of full DMEM, then incubated at 37 °C in 5% CO₂ incubator. The cells were left for 24 h to attach and recover; treatments are followed.

2.7 MTT Assay
After the treatment periods, medium containing drugs was removed, wells washed carefully with PBS for one time and then 100 µl of medium containing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (MP Biomedical, LLC) at 0.5 mg/ml was added to all the microtiter plate wells. The plate was incubated for 3 hours in a CO₂ incubator at 37°C, then the MTT was removed and 100 µl of DMSO were added to dissolve the formazan crystals that were formed. The plate was covered with aluminum foil for 15 minutes and agitated on orbital shaker for 30 seconds. Then, the Optical Density (OD) of the MTT formazan was determined at 550 nm in a microplate reader (Multiskan FC, Republic of Korea).

2.8 Combination studies
Cells were seeded in 96 wells plate at a density of 10,000 cells/well in 100µl of full DMEM. Then chloroformic crude extracts of R. officinalis and ethanolic crude extract of A. sativa were added at different concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/ml) combined with different concentrations of the 5-FU (1.625, 3.25, 6.5, 13, 26 &
52μg/ml). The crude extracts and 5-FU were combined at a constant ratio (3.85:1). The combination of 5-FU with different plant crude extracts were always used at a fixed ratio. All treated plates were incubated for 24 h to 48 h at 37 °C in 5% CO₂ incubator. All experiments were independently repeated at least three times for each plant extract.<ref>11</ref>

2.9 Determination of IC₅₀ and analysis of combinations effect using CompuSyn software

The computer software CompuSyn (The ComboSyn, Inc.) was used for the determination of half maximal inhibitory concentration (IC₅₀), Combination Index (CI) and the dose reduction index (DRI) for the single or combined compounds. This software is based on the median-effect principle of Chou and Talalay<ref>12</ref>.

2.10 Determination of morphological changes of the cells in culture using DAPI stain assay

The Caco-2 cells were grown on clean and sterile coverslips. The coverslips were put into 6-well culture plates and then the Caco-2 cells were seeded into the wells at a density of 4x10⁴–8x10⁴ cells per well. The cells were left overnight to attach, then treated with the crude extracts for the specified time. After treatment, the medium was removed and cells were washed with Phosphate Buffer Saline (PBS) (Biological industries) briefly. The cells were then stained for 10 min using DAPI dye solution (5 μg/ml) to stain DNA at room temperature and then mounted on a slide. Finally, morphological changes were observed under a fluorescent microscope (EVOS XL -AMG, USA) at excitation/emission 357/447 nm<ref>13</ref>.

3. Results

3.1 Antiproliferative effects of different crude plant extracts on Caco-2 Cells

The effect of the different plant extracts on the viability of the colon cancer cell line (Caco-2) was examined. The different crude extracts used in this study showed variable antiproliferative effects against Caco-2 cancer cells. Cells in the exponential growth phase were treated with different concentrations of ethanolic, chloroformic (0, 6.25, 12.5, 25, 50, 100 and 200 μg/ml) or distilled water crude extracts (0, 31.25, 62.5, 125, 250, 500 and 1000 μg/ml) at 24 and 48 h. Viability of cells was determined using MTT assay. At least three independent experiments were done.

3.1.1 Antiproliferative effects of different <i>R. officinalis</i> crude extracts on Caco-2 cells

The inhibitory effect of different concentrations of <i>R. officinalis</i> ethanolic crude extract at different time points on Caco-2 cells was studied. <i>R. officinalis</i> ethanolic crude extract increased the inhibitory effect in dose and time dependent manner (Fig 1A). The inhibitory effect increased from 1.6% to 85.7% as the concentration of <i>R. officinalis</i> increased from 12.5 – 200 μg/ml for 24 h. When the treatment time was increased to 48 h, the inhibitory effect increased from 16.4% to 97.8% as the concentration of <i>R. officinalis</i> increased from 6.25–200 μg/ml. The calculated IC₅₀ is 98 μg/ml and 24.7 μg/ml at 24 h and 48 h, respectively.

The inhibitory effect of different concentrations of <i>R. officinalis</i> chloroformic crude extract at different time points on Caco-2 cells was studied. The crude extract increased the inhibitory effect in a dose and time dependent manner (Fig 1B). The inhibitory effect increased from 11% to 90% as the concentration of the crude extract increased from 6.25 – 200 μg/ml for 24 h. On the other hand, the increase in the treatment time to 48 h increased the inhibitory effect from 16% to 99% as the concentration of <i>R. officinalis</i> increased from 6.25 – 200 μg/ml. The calculated IC₅₀ is 77 μg/ml and 21 μg/ml at 24 h and 48 h, respectively.

The inhibitory effect of different concentrations of <i>R. officinalis</i> distilled water crude extract at different time points on Caco-2 cells was investigated (Fig 1C). <i>R. officinalis</i> distilled water crude extract enhanced the proliferation of Caco-2 cells differently at the different used concentrations for 24 h. In contrast, there was a slight change in the inhibitory effect of <i>R. officinalis</i> distilled water extract when the incubation time was increased to 48 h. The inhibitory effect of <i>R. officinalis</i> distilled water extract increased to 9.2% when the incubation time was increased to 48 h. Additionally, at 48 h incubation, the inhibitory effect increased from 9.2% to 19.4% as the concentration of the crude extract was increased from 250 to 1000 μg/ml.

![Graph A](http://www.florajournal.com)

![Graph B](http://www.florajournal.com)
3.1.2 Antiproliferative effect of different crude extract of *A. sativa* on Caco-2 cells

The inhibitory effects of ethanolic extract of *A. sativa* on Caco-2 cells increased in a dose and time dependent manner (Fig 2A). The inhibitory effect of *A. sativa* ethanolic crude extract on Caco-2 cells increased from 18% to 63% as the concentration increased from 6.25–200 μg/mL at 24 h. Moreover, the inhibition of cell growth was increased from 33% to 82% as the concentration of the crude extract increased from 6.25–200 μg/ml at 48 h. The IC₅₀ for *A. sativa* was 67 μg/ml at 24 h and 11 μg/ml at 48 h.

The inhibitory effect of *A. sativa* chloroformic crude extract on Caco-2 cells increased in a dose and time dependent manner (Fig 2B). The inhibitory effect of *A. sativa* increased from 9% - 61% as the concentration of *A. sativa* chloroformic crude extract increased from 6.25-200 μg/mL at 24 h. Meanwhile, the inhibitory effect increased from 26% - 81% as the concentration of the crude extract increased from 6.25-200 μg/ml at 48 h. The IC₅₀ for *A. sativa* chloroform extract is 98 and 22.8 μg/mL at 24 and 48 hours, respectively.

The inhibitory effect of different concentrations of *A. sativa* distilled water crude extract at different time points on Caco-2 cells was studied (Fig 2C). Generally, *A. sativa* distilled water crude extract increased the proliferation of Caco-2 cells differently at different concentrations for 24 h and 48 h.
Fig (3A) shows the effect of the *P. oleracea* ethanolic extract on colon cancer cells (Caco-2). The results showed that the use of ethanolic crude extract inhibits the growth of Caco-2 in the range of 4.8% to 12.1% at different used concentrations (6.25 – 400 μg/mL) at 24 h. The rate of inhibition of cell growth did not differ significantly when time was increased to 48 h. Fig (3B) shows the effect of the *P. oleracea* chloroformic extract on Caco-2 cells. The results show that the use of chloroformic crude extract inhibits the growth of Caco-2 in the range of 1% to 23% at different used concentrations (6.25 – 400 μg/mL) at 24 h. While the percentage of inhibition ranged from 19.3% to 28.3% at different used concentrations (6.25 – 400 μg/mL) at 48 h.

The inhibitory effect of different concentrations of *P. oleracea* distilled water crude extract at different time points on Caco-2 cells was studied (Fig 3C). Generally, *P. oleracea* distilled water crude extract inhibited the proliferation of Caco-2 cells differently at different used concentrations (31.25 – 1000 µg/mL) for 24 h and 48 h. The maximum inhibitory effect was 15.5% at 250 μg/mL at 24 h and 11.1% at 125 μg/mL at 48 h.

### 3.1.4 Antiproliferative effect of different *P. granatum* crude extract on Caco-2 cells

**Fig 4:** Antiproliferative effects of *P. granatum* A) Ethanolic, B) Chloroformic, and C) DW, crude extract on Caco-2 cells after 24- and 48-hours incubation period; DW, Distilled Water; Values are expressed as mean ± SD of at least 3 independent experiments. *, *P* < 0.05.

The inhibitory effect of different concentrations of *P. granatum* methanolic crude extract at different time points on Caco-2 cells was studied. *P. granatum* methanolic crude extract increased the inhibitory effect in a dose and time dependent manner (Fig 4A). The inhibitory effect increased from 1.1% to 45% as the concentration of *P. granatum* increased from 12.5–200 μg/ml for 24 h. When the treatment time was increased to 48 h, at lower concentrations the crude extract increased the proliferations of cells while increasing the concentration to 25 μg/ml inhibited the proliferation of cells by 6.5%. The maximum inhibitory effect was at concentration of 200 μg/ml, which inhibited the growth of cells by 53.2%. The calculated IC$_{50}$ is 125.5 μg/ml and 120.6 μg/ml at 24 h and 48 h respectively.

The inhibitory effect of different concentrations of *P. granatum* chloroformic crude extract at different time points on Caco-2 cells was studied (Fig 4B). The inhibitory effect of the extract increased from 5.6% to 12.2% as the concentration increased from 6.25–200 μg/mL at 24 h. On the other hand, increasing the time to 48 h increased the inhibitory effect from 2.8%–24.8% as the concentration was increased from 12.5–200 μg/mL. The IC$_{50}$ of *P. granatum* at 48 h is 191.7 μg/ml.

The inhibitory effect of different concentrations of *P. granatum* distilled water crude extract at different time points on Caco-2 cells was studied (Fig 4C). The crude extract increased the proliferation of cells at concentrations 31.25–250 μg/ml at 24 h. As the concentration was increased from 500–1000 μg/ml the inhibitory effect increased from 41.7%–56.5%. On the other hand, the inhibitory effect was increased from 25.5%–88.1% as the concentration of the extract increased from 125–1000 μg/ml at 48 h. The IC$_{50}$ of the *P. granatum* distilled water extract at 24 and 48 h is 786.2 μg/ml and 232.9 μg/ml respectively.

### 3.2 The effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract on normal cells (human lymphocytes)

**Fig 5:** The effect of A) *R. officinalis* chloroformic extract and B) *Avena sativa* ethanolic extract on normal cells (lymphocytes) after 24 h.
The crude extracts with the best inhibitory effect was used to treat normal cell (lymphocytes). Fig (5) shows that the effect of the two extracts was much lower on normal cells as compared to cancer cells (Caco-2 cells).

3.3 Effect of treatment of different crude extracts on Caco-2 cells

Fig 6: Morphological changes of Caco-2 cells after treatment with different crude extracts. A) Caco-2 control cells without treatment after 48 h of seeding; B) Caco-2 cells treated with *R. officinalis* ethanolic extract 200 µg/ml after 24 h of treatment; C) Caco-2 cells treated with *R. officinalis* chloroformic extract 200 µg/ml after 24 h of treatment; D) Caco-2 cells treated with *A. sativa* ethanolic extract 200 µg/ml after 24 h of treatment; E) Caco-2 cells treated with *A. sativa* chloroformic extract 200 µg/ml after 24 h of treatment.

3.4 Effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract on chromosomal condensation in Caco-2 cells

Changes in nuclear morphology and cell distribution were examined by using DAPI stain observing the cells under the inverted microscope. Fig (7A) illustrates the untreated control cells whereas (Fig 7B and 7C) indicate the cells after 24 h treatment with *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract, respectively.

Fig 7: Effect of different crude plant extract on nuclear condensation of Caco-2 after 24 h. Cells were seeded on coverslips as described in materials and methods and after 24 treated with different crude plant and left for 24 h. Cells were then stained using DAPI stain, mounted on a slide using mounting medium and observed under the fluorescence microscope. A) Control Caco-2 without treatment; B) Caco-2 treated with 200 µg/ml of *R. officinalis* chloroformic extract; C) Caco-2 treated with *A. sativa* ethanolic extract 200 µg/ml concentration, magnified 400 times. White arrows indicate cells with nuclear condensation.

3.5 Combination studies

3.5.1 The antiproliferative effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract combined with the chemotherapeutic drug 5-FU

The interaction effect of the two crude extracts with 5-FU on the proliferation of Caco-2 cells was assessed. Crude extracts at different concentrations were combined with different concentrations of the 5-FU. The concentrations used for crude plant extracts were (0, 6.25, 12.5, 25, 50, 100 & 200 µg/ml) and those for 5-FU were (0, 1.625, 3.25, 6.5, 13, 26 & 52 µg/ml). The inhibitory effects data were analyzed by using the CompuSyn software to determine the type of interaction which occurred between *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract combined with 5-FU chemotherapeutic agent individually.

The combination index (CI) value, fraction affected (Fa), dose response index (DRI) and single dose concentrations were calculated by using CompuSyn software. A CI value > 1 means antagonism; CI = 1 means additive effect and CI < 1 means synergism. Fraction affected (Fa) indicates the fraction of cells affected after treatment.
3.5.1.1 The inhibitory effect of *R. officinalis* chloroformic extract combined with 5-FU chemotherapy

Different concentrations of *R. officinalis* chloroformic extract were combined with different concentrations of 5-FU at a fixed ratio of 3.85:1 (Table 3). The concentration that killed 50% of the cells of each drug alone is 98 and 249 µg/mL for *R. officinalis* and 5-FU, respectively. The concentration was reduced to a combination dose of 52.2 µg/mL of both drugs at combination ratio 3.85:1 of both *R. officinalis* and 5-FU respectively. The dose reduction index is 24 and 23 times for *R. officinalis* and 5-FU, respectively (Fig 8). The combination index (CI < 1) was synergistic at all combinations (Table 3).

| Table (3): The inhibitory effect of *R. officinalis* combined with 5-FU on Caco-2 cells after 24 h treatment. |
|------------------|-------------------|-------------------|-----------------|-----------------|
| **Fa** | **R. officinalis** | **5-FU** | **Comb. Dose (µg/ml)** | **R. officinalis** | **5-FU** |
| 0.25 | 46 | 46 | 0.42 | 19.15 | 15.17 + 3.83 |
| 0.5 | 98 | 249 | 0.46 | 52.2 | 41.76 + 10.44 |
| 0.75 | 212 | 1345 | 0.55 | 142.2 | 113.76 + 28.44 |
| 0.9 | 459 | 7270 | 0.68 | 387.6 | 310.08 + 77.52 |

Combination Index (CI) < 1 = 1, > 1 indicates synergism, additive effect, and antagonism, respectively; DRI: The dose-reduction index; Fa: fraction affected.

3.5.1.2 The inhibitory effect of *A. sativa* ethanolic extract combined with 5-FU

Different concentrations of *A. sativa* ethanolic extract was combined with different concentrations of 5-FU at fixed ratio which is 3.85:1 (Table 4). The concentration that killed 50% of the cells of each drug alone is ~ 122 and 249 µg/mL for *A. sativa* and 5-FU, respectively. The combination dose was reduced to ~ 86 µg/mL. At 0.5 Fa, the dose reduction index is 1.8 and 14 times for *A. sativa* and 5-FU, respectively (Fig. 9). The combination index (CI < 1) was synergistic at all combinations (Table 4).

| Table (4): The inhibitory effect of *A. sativa* ethanolic extract combined with 5-FU on Caco-2 cells upon 24 h treatment. |
|------------------|-------------------|-------------------|-----------------|-----------------|
| **Fa** | **A. sativa** | **5-FU** | **Comb. Dose (µg/ml)** | **A. sativa** | **5-FU** |
| 0.25 | 16.3 | 46 | 0.57 | 10.8 | 8.64 + 2.16 |
| 0.5 | 121.9 | 248.6 | 0.63 | 85.8 | 68.64 + 17.16 |
| 0.75 | 912 | 1345 | 0.7 | 682 | 545.6 + 136.4 |
| 0.9 | 6820 | 7270 | 0.78 | 5422 | 4337.6 + 1084.4 |

Combination Index (CI) < 1 = 1, > 1 indicates synergism, additive effect, and antagonism, respectively; DRI: The dose-reduction index; Fa: fraction affected.

4. Discussion

The present study objectives were to assess the antiproliferative effects of the *R. officinalis*, *A. sativa*, *P. granatum* and *P. oleracea* extracts on Caco-2 cells. In addition, investigating the combination effect of 5-FU with the plant extracts: *R. officinalis* (chloroform extract) or *A. sativa* (ethanol extract) on the proliferation of Caco-2 cells.

4.1 Antiproliferative activity of ethanolic crude plants extracts on Caco-2 cells

The results of the present study showed that the ethanolic crude extracts of the different used plants have different antiproliferative effects. The highest antiproliferative effect at 24 h was that of *A. sativa* followed by *R. officinalis* and then *P. granatum*. While, *P. oleracea* showed low antiproliferative effects on Caco-2 cells. The calculated IC50 at 24 h for *A. sativa*, *R. officinalis* and *P. granatum* were 67, 98 and 125.5 µg/mL, respectively.

The results of our study are in agreement with a study of Cheung et al (2007) which showed that *R. officinalis* ethanolic crude extract has antiproliferative effect on human leukemia and breast cancer cell lines [14, 15]. In addition, the results in the present study are in agreement with those of Moore et al (2016) which showed that the *R. officinalis* ethanolic extract drastically decreased colony formation of Caco-2 [16]. Yi & Wetzstein (2011) achieved that the rosemary ethanolic crude extract...
extract decreased cell growth of different colorectal cancer cell line such as SW620 and DLD-1 at a concentration of 31.25 μg/mL and IC₅₀ around 25 μg/mL (48 h) [17]. In the present study, the IC₅₀ of the *R. officinalis* ethanolic crude extract was 98 μg/ml at 24h and 24.7 μg/ml at 48 h which agrees with those of Yi & Wetzelin (2011).

Regarding *A. sativa* ethanolic extract, our results are in accordance with the results of a study by Sato et al. (2016). They investigated the anti-proliferative activities of *A. sativa* against different cancer cell lines like colon (HCT116), lung (NCI-H460) and breast (MCF7). Ethanol extracts indicated higher anti-proliferative activities against HCT116 with inhibition (69.5%), NCI-H460 (75.2%), and MCF7 (84.8%) cells compared with other extracts like methanol and acetone, and ethyl acetate extracts (Sato et al., 2016). In another study which determined the phenolic contents and investigated the activities of *A. sativa* on growth of HepG2 cell line, the results indicated that the *A. sativa* ethanolic extract have antiproliferative and antioxidant capacity [18].

Regarding the ethanolic extract of *P. granatum* peel, a study performed by Malik and Mukhtar (2006) showed that the peel inhibits the growth of human prostate cancer cells (PC3) and MCF-7 in a dose dependent manner [19]. In another study by Khan et al. (2007), they found that 50, 100, and 200 μg/mL of the *P. granatum* peel ethanolic crude extract decreases the human lung carcinoma (A549) cell line viability at rates of 33%, 44%, and 47% [20]. This result agrees to a certain degree with our results which showed that 200 μg/ml of the extract decreased the viability of Caco-2 cells to 45%.

### 4.2 Antiproliferative activity of plant chloroformic crude extract against Caco-2 cell line

The results of the present study showed that the chloroformic crude extracts of the different plants have different antiproliferative effects. The highest antiproliferative effect at 24 h was that of *R. officinalis* followed by *A. sativa* and then *P. granatum*. While *P. oleracea* showed low antiproliferative effects on Caco-2 cells. The calculated IC₅₀ at 24 h for *R. officinalis* and *A. sativa* are 77 and 98 μg/ml respectively. While the calculated IC₅₀ of *P. granatum* 48 h is 191.7 μg/ml.

Despite intensive literature search we couldn’t find any published reports regarding to chloroformic extract of *A. sativa*, therefore to the best of our knowledge this is the first study that investigated the anticancer activity against Caco-2 cell line, it is noticeable from the results we obtained that *A. sativa* chloroformic extract significantly reduced the proliferation of Caco-2.

The results of the present study showed that the rate of inhibition of the chloroformic crude extract of *P. oleracea* ranged from 19.3% to 28.3% at different used concentrations (6.25–400 μg/mL) at 48 h. The results of Mali (2015) are in agreement with our results which showed that the chloroformic crude extract of *P. oleracea* was less efficient or does not have cytotoxic activity against human colon adenocarcinoma cell line [21].

### 4.3 Antiproliferative activity of plant distilled water crude extract against Caco-2 cell line

The results of the present study showed that the antiproliferative effects of distilled water crude extract was low in all used plants extracts except for *P. granatum*. These results come in line with studies involving Chinese herbs and plants, which have been found to have deleterious effects on certain cancer cell lines, and has been long known to induce inflammation and carcinogenesis in different types of cells [22].

### 4.4 The antiproliferative effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract combined with the chemotherapy 5-Fluorouracil (5-FU)

The concept of combination chemoprevention seeks to increase the chemo preventive effectiveness of agents, while decreasing toxicity by dose reduction [23]. In this study, we examined the effects of combining *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract with the chemotherapy 5-FU that is widely used as colorectal cancer treatment locally.

We found that the CI indicates a synergistic effect at combination ratio 3.85:1 of both *R. officinalis* chloroformic extract and 5-FU respectively. When the concentration that killed 50% of the cells of each drug alone is 98 and 249 μg/mL for *R. officinalis* and 5-FU, respectively. These results are in agreement with the result obtained by González et al (2013), who investigated the anti-tumor effect of 5-FU which enhanced by rosemary (*R. officinalis*) extract in both drug resistant and sensitive human colon cancer cells SW620 and DLD-1. The results indicate that the *R. officinalis* sensitizes the 5-FU resistant cells to therapeutic activities of this drug and there was a synergistic effect between 5-FU and rosemary extract. These effects were related to the modulation of the gene expression of thymidylate synthetize and thymidine kinase 1, which are enzymes involved in the mechanism of resistance of 5-FU [24].

### 5. Conclusion

The traditional use of one drug protocol for treatment of cancer is becoming increasingly ineffective. Currently, many research groups use combination therapy, which contain either two or more drugs for treatment of cancer [25]. This combination of anticancer agents may cause synergism, additive or antagonistic effects. The pure natural or synthetic drugs used in western medicine usually affects a single target. On the other hand, crude extracts of natural products contain many components, in various combinations and formulations, aimed at multiple targets.

The present paper found that both extracts chloroformic *R. officinalis* and ethanolic *A. sativa* have concentration dependent antiproliferative effect on Caco-2 cell line. These extracts are selective towards cancer cells than normal cells. *R. officinalis* ethanolic extract has also promising results for its ability to inhibit cell growth of Caco-2. On the other, hand *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract show synergetic effect when combined with 5-FU chemotherapeutic agent at different concentrations and fixed ratio of 3.85:1 and can be considered as a promising strategy for treatment and development of new anticancer agent.

Further studies are needed to determine the mechanisms involved in the synergistic effect; however, results of DAPI study of both *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract showing chromatin condensation which indicate that apoptosis may be induce this property but further studies should confirm it.

### References

2. Van der Jeught K, Xu HC, Li YJ, Lu XB, Ji G. Drug