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RESEARCH ARTICLE

In vitro Apoptotic effects of the medicinal plants Achillea santolina and Raphanus sativus extracts on different cancer cell lines

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ABSTRACT

The extracts of the medicinal plants Achillea santolina and Raphanus sativus have been reported to show anti-cancer effects *in vitro*. However, the cellular and molecular mechanism of these effects are not clear yet. To compare the apoptotic effects of these plant extracts on different cancer cell lines *in vitro*. The phenolic, flavonoids and antioxidant activity were determined in the crude extracts. Then, Caco2 (colon adenocarcinoma) HepG2 (hepatic carcinoma), MCF7 (breast cancer) and the normal WIS (amniotic cell line) were treated in vitro with different concentrations or crude extracts for 72 hours. The half maximal inhibitory concentration (IC50) was detected by MTT assay, while cell cycle and apoptosis were assessed by flow cytometry. The methanolic extract of R. sativus seeds (cultivar Balady) showed higher phenolic content (791.98 mg/d.wt) and higher antioxidant activity (93%) than those of the ethanolic extract of A. santolin (340.23 mg/d.wt) and (72.72%), respectively. R. sativus methanolic extract showed lower flavonoids contents (1.025 mg/g d.wt) than A. santolin ethanolic extract (24.66 mg/g d.wt). Treatment of $CaCO_2$, HepG2, MCF7 an WISH cell lines with *A. santolina* extract showed IC50 of 17.67 µg/ml, 15.1 µg/ml, 42.19 µg/ml and 50.99 µg/ml, respectively. While treatment of the same cell line with R. sativus showed IC50 of 40.77 µg/ml, 27.42 µg/m, 54.16 µg/ml and 86.37 µg/ml respectively. *A. santolina* and *R. sativus* extracts induced similar cell cycle arrest in Caco2 at G1 phase by 42.4%. This study indicates that A. santolina has a potent anticancer activity against the selected cancer cell lines.

Keywords: *Achillea santolina, Raphanus sativus*, Cell lines, Cytotoxicity, Cell cycle, Apoptosis

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INTRODUCTION

Phytochemicals compounds from different plant species have been used to treat cancer (Mahassni and Al-Reemi, 2012; Moreno *et al.,* 2016). The effective compounds, in particular polyphenols and flavonoids are able to target a plethora of cellular and molecular pathways including reactive oxygen species invasion, angiogenesis (Thakur *et al.,* 2014; Singh *et al.,* 2016). In addition to these chemical components, certain plants contain other ingredients, which have been reported to possess anticancer effects (Sangthong *et al.,* 2017). As such the antitumor effect of medicinal plants with different active (ROS), inflammation, cell cycle, apoptosis, ingredients are of paramount significance to explore new drugs.

A. santolina is a flowering wild plant from family Asteraceae, which is distributed particularly in the northern hemisphere (Khan, 1998; Si et al., 2006; Ebadi, 2006). The aerial parts of A. santolina contain volatile oils (Bader et al., 2003; El-Shazly et al., 2004), flavonoids and sesquiterpene lactone (Yusopov et al., 1979). Moreover, this species produces alkaloids, saponins, tannins, resins, sterols, carbohydrates, which have anti-inflammatory effects (Al-Snafi, 2013). The research of antitumor activity is local and not spread on this species in vitro (Ghavami et al., 2010; Elsharkawy, 2014; Choucry, 2016).

R. sativus belongs to family Brassicaceae and its common name is radish which have various medicinal properties. Their seeds have been used in traditional medicine as a diuretic, expectorant and anticancer agent (Graham et al., 1978; Kolonel et al., 2000). The methanol seeds extract of *R. sativus* exhibited significant cytotoxic effect, which led to the isolation and identification of the 4-methylthio-butanyl derivatives with antitumor effects (Kim et al., 2013). The nine ingredients of the extract showed high cytotoxic activity against cancer cell in vitro that consider the *R. sativus* as natural reference to compare with *A. santolina* extract beside cisplatin chemotherapy. Given that mechanisms underlying the antitumor effects of *A. santolina* and *R. sativus* cytotoxic are not clear. This study aimed to compare the anti-proliferative and antiapoptic activities of these two plants against different cancer cell lines types *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Folin Ciocalteau, sodium carbonate, pyrogallol, aluminum chloride, potassium acetate, Quercetin, DPPH (2, 2 diphenyl-1-picrylhydrazyl), ammonium molybdate, sodium phosphate, sulphoric acid, dimethyl sulfoxide (DMSO), MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Company, (USA). RPMI 1640 medium, Phosphate Buffered Saline (PBS), FBS, trypsin- EDTA purchased from Lonza, company, (UK). Annexin V-FITC, (PI), binding buffer 1X (BD Pharmingen), Triton ™ X100, purchased from BD company, (USA). RNase A (BIOBASIC, CANADA).

Plants collection and extraction

R. sativus seeds (cultivar: Balady) were obtained from Agriculture Research Center, Giza, Egypt. The wild plant *A. santolina* was collected from Mersa Matruh (North West Coast of Egypt) and identified in Ecology Department, Faculty of Science, Tanta University. Aqueous ethanol (80 %) was used to extract the shoots (leaves and flowers) of *A. santolina* according to Ghavami *et al.* (2010). Methanol (96 %) solution was used for extraction of radish seeds after grinding to fine powder according to Parekh *et al.* (2005). All extractions were kept for 5 days at room temperature. The extracts were filtrated and allowed to evaporate by rotary evaporator and then stored in -20^{o}C as powder until use.

Determination of phenolic compounds

The total phenolic content was determined according to Malick and Singh, (1980). The 10mg crude plant extracts dissolved in dist. H2O, then (0.5 ml) of stock were pipetted into test tubes and the volume was completed to 3 ml with distal water. Then, 0.5 ml of Folin reagent was added to each tube and incubated for 3 minutes. Two ml of 20% Na2CO3 solution was added to each tube, mixed and incubated for 1 hr. in dark at room temperature. The standard curve of pyrogallol was prepared by using different concentrations from 0.0 to 0.1mg/ml and the absorbance at 650 nm was measured against blank using Cole Parmer Spectrophotometer 1200 (Unico, USA). The data were expressed as milligram / gram. dry weight (g. dwt).

Determination of flavonoids

The total flavonoids content was determined according to the colorimetric method described by Chang *et al.* (2002). Plant extract solutions (0.5 ml) were pipetted into test tubes, then 1.5 ml ethanol 95% was added. Then, 0.1 ml of 10% aluminum chloride and potassium acetate (0.1 ml) of 1 M was added to tubes and mixed well and 2.8 ml distal water was added in all tubes and left at room temperature for 30 min. In case of blank, aluminum chloride was replaced by dist. water. Quercetin was chosen as a standard using a ten-point standard curve (0.0-0.1 mg/ml). The absorbance of each mixture was determined at 415 nm using Cole Parmer Spectrophotometer 1200 (Unico, USA). The data were expressed as milligram quercetin equivalents (QE)/g.

Determination of antioxidants activity using DPPH

The extract solution (0.1ml) was added to 3.9 ml of DPPH solution. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 1 hr. The decrease in absorbance was monitored at 517 nm using Cole Parmer Spectrophotometer 1200 (Unico, USA) after 1 hr. of the reaction. The blank

consisted of 0.1 ml of methanol or ethanol (extraction reagent) and 3.9 ml of DPPH solution. The percentage of scavenged DPPH was calculated from the following equation (Sannigrahi *et al.,* 2009).

DPPH scavenging % = (Ao – As/Ao) X 100 Where Ao: is the absorbance of the blank As: is the absorbance of sample

Determination of the total antioxidant using phosphomolybdate assay (PMA)

The total antioxidant content was determined according to the method described by Kumaran, (2007). The extract solution (300 µl) was added to 3 ml of mixture of (0.05 g sodium phosphate, ammonium molybdate (0.05g) and sulphoric acid (0.33 ml) each dissolved in 10 ml dist.H2O. The tubes were then incubated in water-bath at 95 ºC for 30 min. After cooling of the solution, the absorbance was measured at 765 nm using Cole Parmer Spectrophotometer 1200 (Unico, USA).

Cancer cell lines

Normal human cell line and cancer human cell lines were obtained from VACSERA, Dokki, (Egypt). Three types of cancer human cell lines Caco2 (epithelial colorectal adenocarcinoma), MCF7 (human breast carcinoma) and HepG2 (human hepato carcinoma) were used. The normal cell line WISH (derived from normal amnion) was used as a control cell. Cell lines were grown in tissue culture T-75 flasks containing complete RPMI 1640 medium supplemented with 10% FBS, 1% (wt/vol) L-glutamate and 50 µg/ml gentamycin sulphate at 37ºC in 5% CO2. The cells were maintained by routine sub culturing in T- 75 flasks tissue culture flasks. Cell viability was sassed by trypan blue exclusion assay.

Determination of IC50 concentrations

Adherent cells were harvested with 2 ml trypsin-EDTA 0.25% then counted by hemocytometer. After that cells were seeded into 96-well plates in 100 µl (10^4cell/well) for each well, the cells were then incubated in 5% CO2 at 37°C incubator for 24 hrs. Stock solutions of *A. santolina* and *R. sativus* dried extracts were prepared by dissolving 1mg of the extract powder in 1ml DMSO, while cisplatin concentration (ref. drug) was (1mg/ml). Serial dilutions (0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 µg/ml) were prepared and 10 µl of each dilution was used in each well for treatment of cell lines. The plates were incubated for 72 hrs. at 37°C and in 5% CO_2. After 72 hrs of incubation, the medium was discarded from the plates and then the plates were washed with 100 µl PBS, MTT assay was performed for determining the cytotoxicity by adding 25 µl of MTT (5 mg/ml) to the each well and the plates were then incubated for 4 hrs. Then, cells were washed with PBS and 150 µl DMSO were added to each well. The absorbance was measured at 545 nm using Plate Reader. The IC50 analysis were performed using Graph Pad Prism 6.0

Cell cycle analysis

For cell cycle analysis, the cells were cultured in 6 well plates. Each well was filled with 3 ml of fresh medium containing 1×10^5/ml cells. After treatment with 1/10 of IC50 concentration, the seeded cells were incubated for 72 hrs. Then the cells were collected by cell scraper, and prepared for cell cycle measurement protocol. The cells were centrifuged and washed twice with cold 1× PBS. The cell pellets were fixed with 5 ml of 70% ethanol, then stored at -4º C for 48 hrs. After that, cells were resuspended in 200 µl of PI/ Triton × 100 staining solution (1000µl of 0.1% triton+ 40µl PI + 20µl RNAse). The cells were then incubated at 37ºC for 15 min. and transferred onto ice. The tubes were gently vortexed and kept away from the light. Finally, the samples were measured using flow cytometry (BD FACSCanto™Π).

Apoptosis assay

Coco2 cell lines were used for determination of the apoptosis. 3 ml of fresh complete medium containing 1×10^5/ ml cells were seeded in each well of 6 well plates and incubated for 24 hrs, then cells were treated with 1/10 of IC50 of *A. santolina*, *R. sativus* and Cis for 72 hrs, and then cells were harvested with a cell scraper and centrifuged at 10,000 xg at 4ºC for 15 min. The pellet was washed once in cold PBS, and centrifuged at 1800 rpm for 5 min. Then resuspended in 1X Binding Buffer at 1 x 10^6 cells/ml. 5µl Annexin V was added to 100µl of the cell suspension. The cell suspension was

incubated 10-15 minutes at room temperature, then cells were washed in 1X Binding Buffer and resuspended in 200µl of 1X Binding Buffer. Then, 5µl staining propidium iodide solution was added. Finally, the samples were measured using flow cytometry (BD FACSCanto™Π).

Statistical analysis

The data is expressed as Mean ± SD. The statistical analysis of cytotoxicity data and IC50 analysis were performed using Graph Pad Prism 6.0.

RESULTS

R. sativus phenolic activity is more pronounced than *A. santolina*

The results in Table 1 show that *R. sativus* extract has higher phenols content (791.98 mg/g.dwt) while *A. santolina* has a higher flavonoids content (24.664 mg/g dwt).

Estimation of the antioxidant activity

The percentage of antioxidant activity as determined by DPPH and PMA is shown in Figure 1. *R. sativus* ethanolic extract exhibited higher antioxidant activity (93%) than that of *A. santolina* (72.72%). On the other hand, the total antioxidant activity of the crude extract determined by PMA showed that the antioxidant activity was higher in *A. santolina* extract (15.8 mg/g ≈ ascorbic acid equivalent mg/g. dwt) than of that of *R. sativus* (8.64 mg/g ≈ ascorbic acid equivalent mg/g. dwt).

Determination of IC50 of the plant extracts

The IC50 of *A. santolina, R. sativus* and Cis are shown in Fig. 2. The *A. santolina* extract showed lower IC50 as compared to *R. sativus* and cisplatin IC50 in cancer cell lines (Caco2 & HepG2). While *A. santolina* IC50 was similar to cisplatin IC50 value in MCF7 cell line as shown in Table2. The 1/10 of IC50 concentration of *A. santolina, R. sativus* and Cis were used in cell cycle cell and apoptosis cell treatment.

Cytotoxicity assessment

The cytotoxic effects of *A. santolina*, *R. sativus* extracts on the different cell lines compared to Cis are shown in Figure 2. The WISH cell line (normal cell line) was not significantly affected by treatment with the plant extracts *A. santolina* and *R. sativus* at (5 µg/ml) exhibited cell viability by 88.11% and 65.4%, respectively. While cisplatin shows high cytotoxic effect 54% viability cells was observed at the same concentration (Figure 2A). Treatment of Caco2 cell line with both extracts (5 µg/ml) *A. santolina* and *R. sativus* showed high cytotoxic effect 91.83 % and 94.02 % compared to exhibited by Cis has approach to (95.62%) (Figure 2B**)**. Treatment of MCF7cells with (5 µg/ml) concentration of *A. santolina* and *R. sativus* extracts showed 82.06% and 62.13% dead cells, respectively (Figure 2C). The same concentration of cisplatin drug exhibited 80.39% of cells death. Treatment of HepG2 cells with (5 µg/ml) of both plant (*A. santolina* and *R. sativus*) extracts and cisplatin showed similar cytotoxic effect 58.53%, 58.19% 57.43 % Dead cells, respectively (Figure 2D).

Effect of plant extracts on cell cycles of the tumor cell lines

The DNA content of cancer cell lines (Caco2, MCF7 and HepG2) and normal cells (WISH) during cell cycle after treatment (for 72 hrs.) with 1/10 of IC50 of *A. santolina, R. sativus* extracts or Cis was determined. The graphs of fractional DNA content (PI fluorescence, X-axis) versus cell counts (Y-axis) are displayed in Figure 3. In case of WISH (normal cells), treatment with 1/10 of IC*R*50*R* of *A. santolina* or *R. sativus* extracts resulted in 40% and 23.2% cell accumulation in G2/M phase, while Cis treatment showed 75.5% arrest at G0/G1 as shown in (Figure 3B-D). Concerning the Caco2 cell line, treatment with *A. santolina* and *R. sativus* extracts induced similar cell cycle arrest at G0/G1 phase (42.4%) compared to normal cell line (WISH) (Fig. 3 G&H). However, Cis treatment exhibited 39.9% cell cycle arrest at G0/G1 phase (Figure 3F).

In case of MCF7, the cell line treated with *A. santolina* extract showed 49% cell cycle arrest at G0/G1 phase (Figure 3K), while treatment with *R. sativus* extract or Cis showed similar cell cycle arrest 61.7 % and 63.9%, respectively at S phase (Figure 3 J&L). With regard to HepG2 cell line, the untreated cells showed 55.5% bulk at G2/M, while treatment with *A. santolina* extract and Cis drug increased cells bulk in sub-G0 to 69.9% and 44.2%, respectively.

Table 1. The concentration of total phenols and flavonoids determined in the crude extracts of *A. santolina* and *R. sativus.*

Compound	*A. santolina* Mean ± SD	*R. sativus* Mean ± SD
Phenols contents mg/g. dwt	340.23 ± 8.66	791.98 ± 79.85
Flavonoids compounds mg/g. dwt	24.66 ± 1.97	1.025 ± 0.077

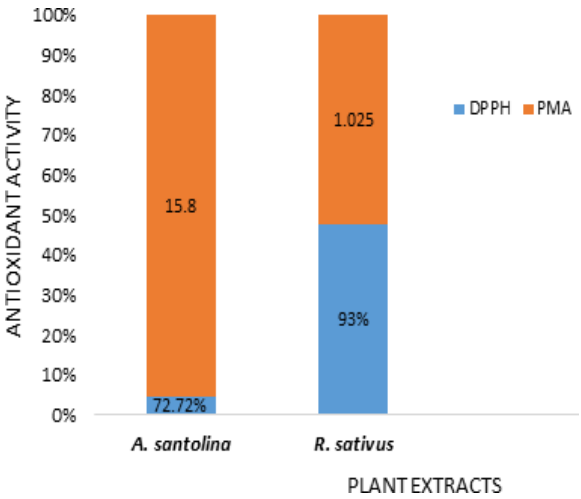

Figure 1. Antioxidant activity of *A. santolina* and *R. sativus* extracts

Table 2. The IC50 µg/ ml of plant extracts determined for the different cell lines.

Cell line	*A. Santolina* IC50 µg/ml	*R. sativus* IC50 µg/ml	Cisplatin IC50 µg/ml
Wish	0.5099	0.8637	1.8090
Caco2	0.1767	0.4077	0.1280
MCF7	0.4219	0.5416	0.05858
HepG2	0.1512	0.2742	0.2398

HepG2 cells treated with *R. sativus* extract showed 58.1% cell cycle arrest at S-phase (Figure 3N-P).

Apoptosis

The early apoptotic cells detection (FITC-Annexin V positive and PI negative) is shown in Fig. 4, which demonstrates apoptotic percentages of the cell line (Caco2) treated with 1/10 of IC50 of plant extracts and Cis drug. The Caco2 cells treated with 1/10 of IC50 of *A. santolina* and *R. sativus* extracts showed 9.6% and 18.2% apoptosis, respectively, while 24.3% apoptosis was shown with Cis drug treatment.

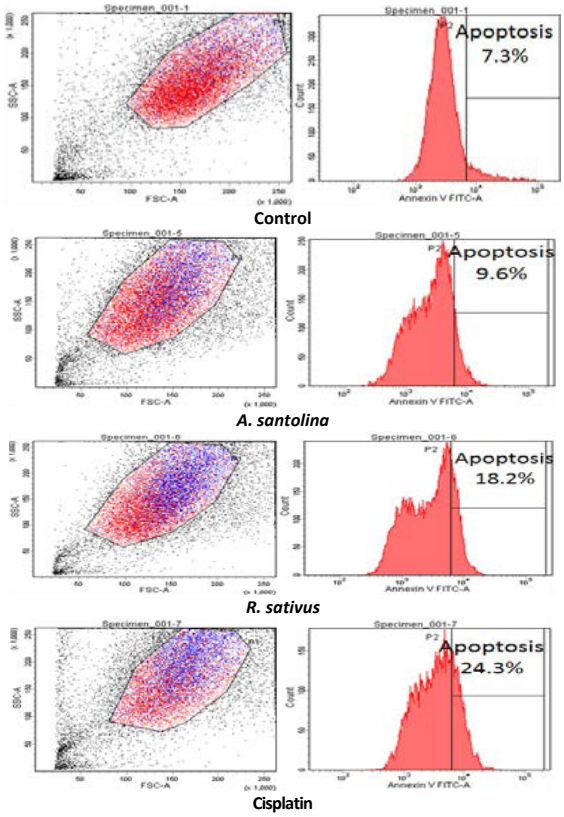

Figure 4. apoptotic effect measured by flow cytometry of 1/10 of IC50 concentration of *A. santolina, R. sativus* and cisplatin on Wish and $CaCO_2$ cell line after incubation in CO_2 incubator for 72 hrs.

DISSCUSSION

The results of this study showed that the ethanolic and methanolic extracts of the *A. santolina* and *R. sativus* contain high total phenols and flavonoids content. High antioxidants activity of *A. santolina* ethanolic extract could be due to its higher flavonoids content. In contrast, the higher antioxidant activity present in *R. sativus* methanolic extract could be explained by its high content of phenolic compounds. This result is in consistence with the finding of Kim *et al.* (2015) and Jin *et al.* (2016).

Cytotoxicity analysis revealed that *A. santolina* ethanolic extract showed higher cytotoxic effect on Caco2 and HepG2 cancer cells at low concentration compared to that of *R. sativus* methanolic extract. Since the *A. santoilna* extract has smaller IC50 than that of *R. sativus* extract and Cis drug, its IC50 concentration showed higher inhibitory effect on the proliferative activity of the Caco2 and HepG2 cancer cell lines more than on MCF7 cell line.

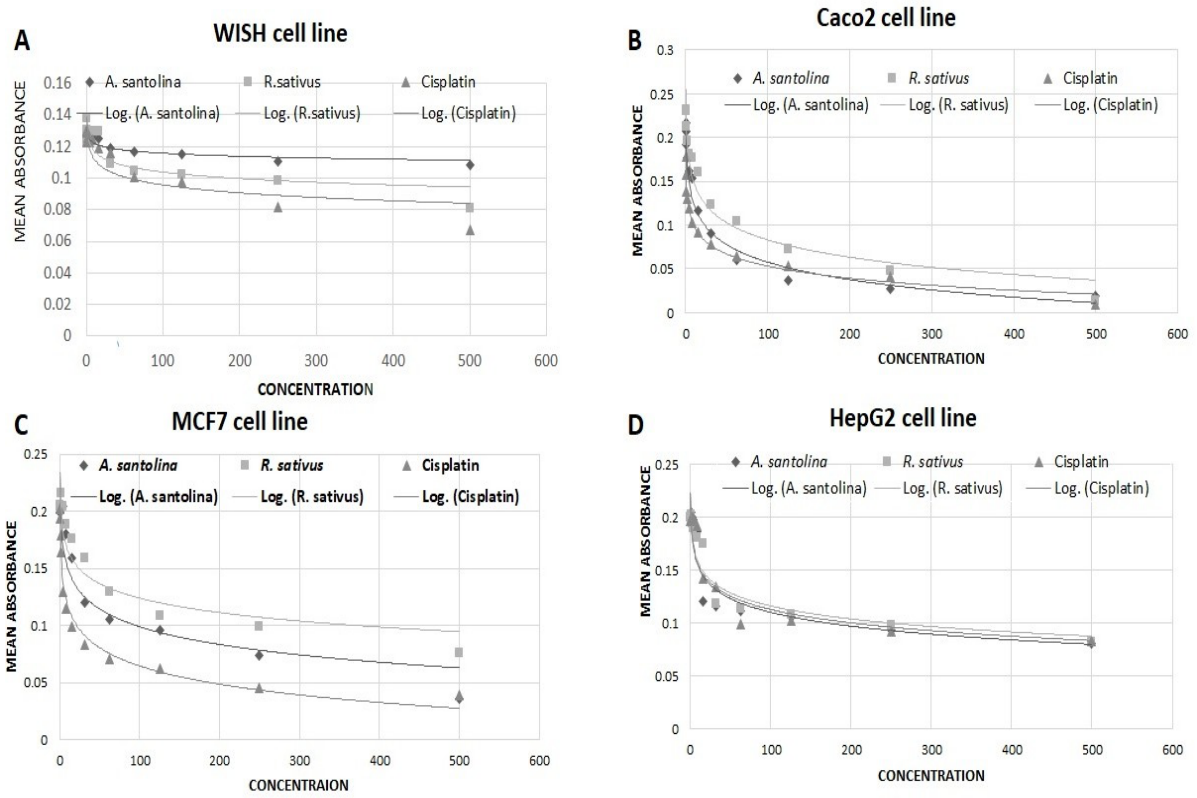

Figure 2. Cytotoxic effects of treatment with different concentrations (*A. santolina*, *R. sativus* and Cis) on WISH normal cell, Caco2, MCF7 and HepG2 cancer cells.

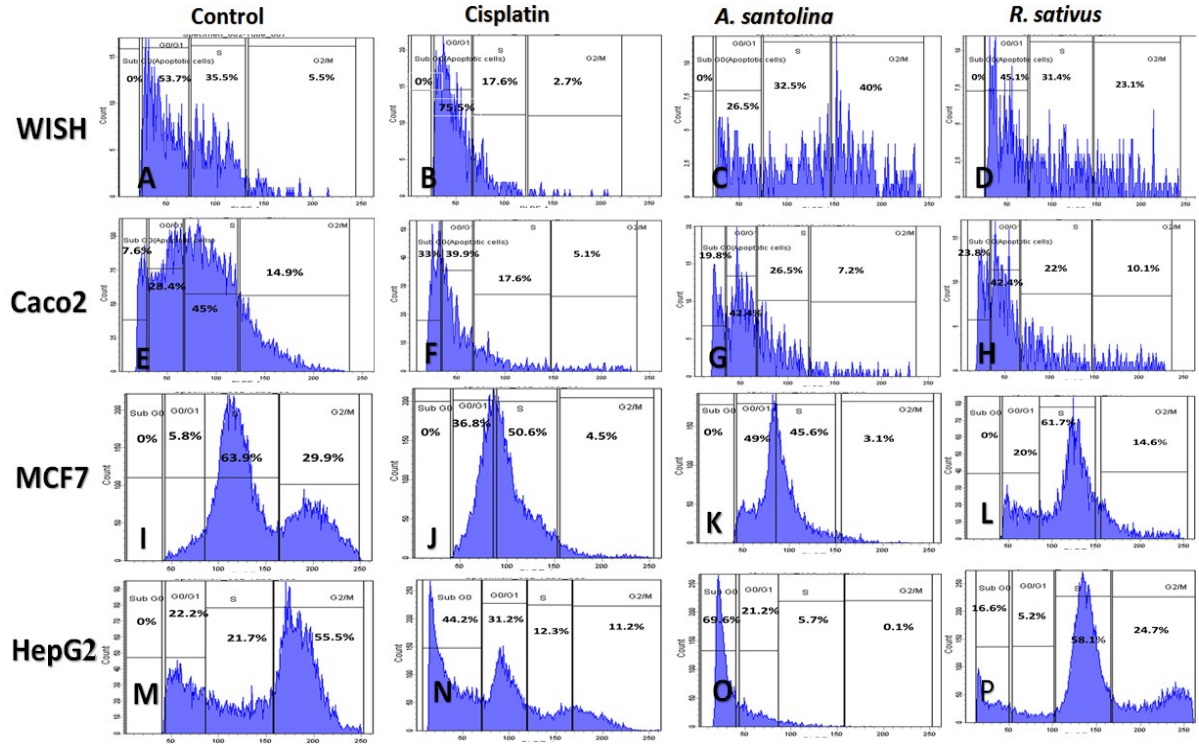

Figure 3. Cell cycle and fractional DNA content in control and Wish (normal) cell line, Caco2, MCF7 and HepG2 cancer cell lines treated with 1/10 of IC_{50} of A. santolina, R. sativus extracts and cisplatin drug after 72h of incubation.

This effect would be due to its higher flavonoids content and antioxidants activity. It has been shown that flavonoids, phenolic compounds and antioxidants in the volatile oil of *Achillea* species play a role in anti-cancer and anti-inflammatory effects (Abd-Alla *et al.,* 2016). Recent studies showed that extracts of *Achillea* species greatly inhibited colon, breast and liver cancers (Elsharkawy, 2014; Choucry, 2017). The free radical scavenging in *R. sativus* seeds extract has been shown to have anti-proliferative effect on different cancer cell lines (Salah-Abbes *et al.,* 2010).

Cell cycle distribution and apoptosis analyses of the used cancer cell lines treated with 1/10 of IC50 of *A. santolina*, *R. sativus* extracts and Cis drug showed a considerable effect on DNA content and cell cycle arrest at G1/S phase. The 1/10 of IC50 of *A. santolina* and *R. sativus* enhanced the cell arrest at the G1 checkpoint in CaCO2 cell line. MCF7 cells treated with 1/10 of IC50 *A. santolina* induced 49% cell arrest at G0/G1. This also may due to higher antioxidant activity of flavonoids and phenolic compounds on G1 checkpoints. This result is in agreement with recent *in vitro* studies performed on different cancer cell lines which were treated with extracts of some medicinal plants (*Glycyrrhiza glabra* and *Lepidium sativum*) which contain high levels of polyphenols and flavonoid compounds. The plant extracts showed strong effect on G1 checkpoint arrest and induced high effect on cell apoptosis (Bortolotto *et al.,* 2016), and also high apoptotic effect on breast cancer (Mahassni and Al-Reemi, 2013). However, our prove results effects similar in previous studies on Caco2 cell line of poly phenols on cell cycle G1 arrest and induce apoptotic effect on colon cancer cells (Huang *et al.,* 2017; López de las Hazas *et al.,* 2017).

The S- and M- phases are rigorously ordered in cyclic process, which allows correct duplication of DNA in the dividing cells without accumulating genetic abnormalities (Pucci *et al.,* 2000). Treatement of MCF7 and HepG2 cell lines with 1/10 of IC50 of *R. sativus* induced S-phase arrest checkpoints. Similarly, 1/10 of IC50 treatment of Cis drug induced arrest in S-phase checkpoint in MCF7 cancer cells. The effect caused by 1/10 of IC50 of *R. sativus* extract could be due to the higher phenolic compounds. This result is consistence with what have been obtained by Luo *et al.* (2017). They showed that phenolic compounds of the natural extracts induced cell arrest at S-phase in breast and colon cancer. The same finding has been found with HepG2 cancer cell line (Saleem *et al.,* 2002). Other study showed G2/M phase cell arrest with sulforaphane compound found in *R. sativus* methanolic extract (Pledgie-Tracy *et al.,* 2007). The treated HepG2 cells with 1/10 of IC50 of *A. santolina* induce sub G0 phase apoptosis (Sánchez- Carranza *et al.,* 2017).

The induction of apoptosis and necrosis in Caco2 cells treated with the extracts of *A. santolina* and *R. sativus* demonstrated that the 1/10 of IC50 of *R. sativus* treatment sowed higher apoptotic effect (18.2%) than treatment with 1/10 of IC50 of *A. santolina* extract 9.6%. This could be due to effect of higher phenolic content on cells at G1 phase which is the state preceding DNA replication in which factors such as cellular conditions (metabolism, signaling and cell size) influence cell cycle progression (Pucci *et al.,* 2000).

CONCLUSION

A. santolina and R. sativus crude extracts both contain polyphenols, flavonoids and antioxidants with different ratios, which inhibit proliferation of the cancer cells as colon adenocarcinoma (Caco2), breast cancer (MCF7) and liver cancer (HepG2) *in vitro*. The 1/10 of IC50 of *A. santolina* raised the percentage of G1-phase arrest in Caco2 and S-phase in MCF7 compared to cisplatin chemotherapy. It also increased apoptosis of the Caco2 cell line. This indicate that these natural extracts have potential effects on different cancer types comparable to cisplatin drug.

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