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Research Article

Antiproliferative and proapoptotic effects of leaf, fruit and stem extracts of *Pistacia lentiscus* on human colon and gastric cancer cell lines.

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ABSTRACT

The use of natural products of plant base has been considerable interest in the treatment of human diseases specifically cancer. Therefore, the purpose of this study was to determine whether methanolic extracts obtained from *Pistacia lentiscus* collected in the north of Tunisia, contain components capable of inhibiting the growth of the human colon carcinoma CaCo2 cell line and the human gastric cancer AGS cell line. Leaves, fruit and stems of this plant were extracted with 80% methanol and used for the cell viability, cell morphology, apoptosis, and cell cycle arrest experiments.

Concerning the two cell lines, leaf and fruit extracts caused significantly decreased cell viability, in a dose-dependent fashion, with cytotoxic effect close to 50%. Flow cytometry and other analyses showed that extracts induced cell apoptosis after 24 h of treatment. The leaf extract was more potent than fruit and stem extracts, showing a 57% apoptosis in the CaCo2 cell line and 53% in the AGS cell line. Furthermore, cell cycle alteration was evaluated. The leaf and fruit extracts induced a G_0/G_1 accumulation in AGS cell lines, whereas the stem extract showed an accumulation of cells in the G_2/M phase. Treatment of CaCo2 cells with fruit and stem extracts for 24 h led to cell arrest in the G_2/M phase at a concentration of 400 µg/ml, however a treatment with the leaf extract caused a significant arrest of the cells in the G_0/G_1 phase. These results suggest that due to the existence of bioactive components, methanolic extracts of *P. lentiscus* have significant cytotoxic effects against human colon carcinoma CaCo2 cells and human gastric cancer AGS cells.

Keywords: *Pistacia lentiscus*; growth inhibition; apoptosis; cell cycle; colon cancer cells; gastric cancer cells.

INTRODUCTION

Cancer is the second most common cause of death following cardiovascular diseases. Recently, several natural anti-cancer drugs have drawn considerable attention as potential agents for adjuvant brain tumor therapy[1,2]. Moreover, there is growing interest in the use of plants in the maintenance of human health.

It is already known that plants contain a wide variety of components that may have biological activities including anti-cancer activity[3] and large numbers of anti-cancer drugs (over of 60%) are derived from natural sources, such as vincristine, irinotecan and etoposide from herbal sources, and doxorubicin,

bleomycin and dactinomycin extracted from marine sources[4].

Most of these bioactive substances exert their chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumor cells has become an indicator of the tumor treatment response; in such case a plant derived-bioactive substance was employed to reduce and control human mortality due to cancer[5,6].

Pistacia lentiscus is an evergreen member of the Anacardiaceae family, producing bright red globose berries. It is largely distributed in "extreme" ecosystems of the Mediterranean basin[7], where it is used as a food ingredient.

It is also used as an antibacterial[8] and anti-ulcer agent[9]. The leaves are extensively used in folk medicine for the treatment of eczema; diarrhea and throat infections[10].The aerial parts of the plant also have a hypotensive action[11]. Flavonoid glycosides have been isolated from the arial parts of P. *lentiscus*[12] and the polyphenolic composition of the leaves has also been reported[13].

In the present study, our aim was to investigate *in vitro* the antiproliferative effect of methanol extracts of the leaves, fruits and stems of *P. lentiscus* on a human gastric carcinoma cell line (AGS) and on a human colon adenocarcinoma cell line (CaCo2). In addition cell morphology, DNA fragmentation, cell cycle progression, and cell apoptosis induction were examined in response to different concentrations of *P. lentiscus* methanol extracts.

MATERIALS AND METHODS

Plant collection and Preparation of *Pistacia lentiscus* extracts

The leaves, fruits and stems of *P. lentiscus* were collected in November, in forested areas from Bizerte which situated in the north of Tunisia. Identification was carried out by Pr. Ouni (Department of Botany, Faculty of Sciences. University of Carthage.Tunisia), according to the flora of Tunisia[14]. A voucher specimen (Pl-11-12) has been kept in our laboratory for future reference.

The air dried and finely ground aerial parts of *P. lentiscus* fruit, leaves and stems (500 g) were successively extracted with 80% methanol at room temperature.

The homogenate was continuously stirred for 24 h and centrifuged at 1500 rpm for 10 min. The pellet was extracted twice and the extract was evaporated to dryness under reduced pressure at 35˚C to obtain a dry powder methanolic extract.

Cell culture

AGS human gastric carcinoma cells (ATCC CRL-1739, Rockville, MD, USA) and CaCo-2 human colon adenocarcinoma cells (ATCC 169, DSMZ collection) were maintained in Ham's F12K medium (Invitrogen, Cergy-Pontoise, France) and in modified Eagle's medium (Invitrogen), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 5 units/ml of penicillin and 5 µg/ml of streptomycin (Sigma, Saint-Quentin-Fallavier, France) at 37°C in a 5% CO_2 humidified atmosphere.

Cells were maintained as a monolayer in 75 cm^2 culture flasks.

MTT bioassay

The cytotoxic effect of *P. lentiscus* methanolic extracts of leaves, fruit and stems on CaCo2 and AGS cells was evaluated using the MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sigma bioassay).

This assay detects the reduction of the yellow dye MTT by mitochondrial succinate dehydrogenase to a blue formazan product, which reflects the normal functioning of mitochondria and hinders cell viability[15].

The cells were seeded in triplicate for each experimental condition in 96-well microtiter plates (100 µl/well) at a concentration of $2x10^3$cells/cm^2. After 48 h, the cultivated cells were exposed to various concentrations of methanolic extracts (400, 40 and 4 µg/ml) prepared in PBS. A control group received the same amounts of PBS alone.

After the treatment, normal culture medium was substituted with 100 µl of fresh media and 20 µl of MTT reagent (5 mg/ml in PBS), except for the three cell-free blank control wells.

Cells were maintained in 5% CO_2 at 37˚C and total humidity for 4 h. Subsequently, the MTT solution was replaced with 100 µl DMSO and 12 µl of Sorensen buffer (0.1 M NaCl, 0.1 M glycine adjusted to pH: 10.5 with 1M NaOH) and incubated for 15 min at 37˚C.

The optical density of the wells was measured at 570 nm using a spectrophotometric plate reader (SPECTROstar Nano).

The growth and viability of the cells were determined using the formula: % viability = (optical density of sample/optical density of control) x100

Cell cycle and apoptosis assay

The propidium iodide (PI) flow cytometric assay was used for cell cycle and apoptosis analysis. Briefly, $5x10^4$ cells were cultured in 24-well plates and 24 h

later treated with *P. lentiscus* methanolic extracts of leaves, fruit and stems at 400 µg/ml or the vehicle (PBS) for 1 day; the positive control was treated with staurosporine (1 mM). Both the floating and adherent cells were collected and detached with trypsin/EDTA and washed twice with PBS prior to staining using a fluorochrome solution (0.1% sodium citrate (wt/v), 0.1% Triton X-100 (v/v), 50 mg/l PI in deionized/distilled water), then the cell samples were incubated at 4°C in the dark for at least 4 h[16]. A BD CantoII flow cytometer (BD Biosciences) was used for data acquisition and analysis.

Nuclear staining with DAPI

After treatment, cells previously seeded on glass coverslips were quickly washed with ice cold PBS, fixed in 70% ethanol at -20°C for 5 min with the DNA- specific fluorochrome DAPI (Invitrogen). Excess DAPI was removed by PBS washes and coverslips were mounted on glass slides with fluoromount G mounting medium (Clinisciences). Observation of the cells and acquisition of fluorescence images were observed using a NIKON Eclipse TE2000 epifluorescence microscope (NIKON, Champigny sur Marne, France) equipped with a digital camera. Cells with condensed chromatin or picnotic fragmented nuclei were considered as apoptotic.

Cell morphology

The effect of *P. lentiscus* extract on cell morphology was monitored as follows: $5x10^6$ cells were seeded in 12-well plates and treated with methanolic extracts (leaves, fruit and stems) at 400 µg/ml for 24 h. Cellular imaging was performed with a phase-contrast light microscope equipped with a digital camera (Zeiss, Germany).

Statistical analysis

The statistical significance of the differences between the control and treated groups was determined by one-way analysis of variance (ANOVA). $p<0.05$ was considered to be statistically significant.

RESULTS

***Pistacia lentiscus* methanolic extracts inhibit cell viability of human colon and gastric cancer cells**

CaCo2 and AGS cells exposed to three different concentrations of the methanolic extracts of leaves, fruit and stems exhibited a dose-dependent reduction of the percentage of cell viability determined by MTT assay, revealing the preservation of cell viability in the presence of extracts (Figure1). Leaf and fruit methanolic extracts from *P. lentiscus* displayed selective cancer cell line cytotoxicity, causing a significant growth inhibition in a dose-dependent fashion, with percentage close to 50% at 400 µg/ml.

When the methanolic leaf extract was compared to the fruit and stem extracts, the leaf extract appeared to be the most potent with 54% and 50% ($P<0.001$) values at 400 µg/ml against gastric and colon cancer cells, respectively.

The stem extract had the lowest cytotoxic action at the three concentrations tested, the values of 50% inhibition of the stem extract being greater than 400 µg/ml for both CaCo2 and AGS cells, with 70% and 79% viability at a concentration of 400 µg/ml ($P<0.001$) for AGS and CaCo2 cells, respectively.

All investigated extracts of *P. lentiscus* exerted selective dose-dependent inhibition of cell viability on AGS and CaCo2 cells (Figure 1).

***Pistacia lentiscus* methanolic extracts induce apoptosis in human colon and gastric cancer cells**

As all of the samples at a concentration of 400 µg/ml caused a significant lack of viability of CaCo2 and AGS cells, we carried out further investigations to determine whether these plant extracts also induced apoptosis in these cell lines.

Treatment of CaCo2 and AGS cells with methanolic extracts of leaves, fruit and stems at 400 µg/ml for 24 reached 57% cell death, respectively. To determine the features of CaCo2 and AGS cell death, the propidium iodide (PI) incorporation was carried out by flow cytometry. In the control group, the cell death ratio was approximately 4.7 and 3.2% in CaCo2 and AGS cell lines, respectively (Figure 2). When CaCo2 cell lines were treated with the different extracts at 400 µg/ml, the number of apoptotic cells increased significantly ($p<0.001$), to 57% with leaf extracts, 21% with fruit extracts, and 17% with stem extracts.

For AGS cells treated with 400 µg/ml of the different extracts, the apoptotic rate was 53 % with leaf extracts, 41% with fruit extracts, and 35% with stem extracts.

In both cell lines, leaf extracts (as well as staurosporine which served as a positive control) exhibited the highest proapoptotic effect. Fruit extracts displayed less pronounced cytotoxicity and apoptosis. Stem extracts had the lowest cytotoxic and proapoptotic effects.

Apoptosis was confirmed by fluorescence imaging DAPI staining was performed on AGS and CaCo2 cells treated with leaf, fruit and stem methanolic extracts. After 24 h of incubation with the plant extracts, an increased number of cells displaying irregular edges of the nuclei, chromatin condensation and picnotic nuclei were observed in treated AGS

cells, while round, clear edged, uniformly stained nuclei were noted in the untreated control (Figure 3).

***Pistacia lentiscus* methanolic extracts induce morphological changes in human colon and gastric cancer cell lines**

After a 24 h treatment with the methanolic extracts of *P. lentiscus* leaves, fruit and stems at 400 µg/ml, alterations in AGS and CaCo2 cell morphology were observed. Compared with untreated controls, the cells exhibited a less regular shape and adhesion and spreading were reduced. Cells were shrunken, round and some detached from the cell culture substratum (Figure 4).

***Pistacia lentiscus* methanolic extracts induce a cell cycle arrest in human colon and gastric cancer cell lines**

When AGS cells were treated with 400 µg/ml of leaf extract from *P. lentiscus*, a significant increase of approximately 8-10% of the cells in the G_0/G_1 phase were observed compared to the untreated negative control, at a similar level to that induced by staurosporine used as a positive control. (Figure 5A). This increase was associated with a concomitant decrease of cells in the G_2/M phase. However, an increased accumulation of cells was observed in the G_2/M phase: from 28% in the untreated cells to 35% in the cells treated with 400 µg/ml of stem methanolic extract.

Moreover, in CaCo2 cells, the *P. lentiscus* methanolic fruit and stem extracts did not induce G_1 arrest but rather a significant accumulation of cells in the G_2/M phase ($p<0.05$). The percentages of the cells in G_2/M phase increased from 19% in untreated control cells to 24% and 27% in cells treated with stem and fruit extracts, respectively.

DISCUSSION

The natural products play an important role in drug discovery. Presently, the use of active compounds extracted from herbs in the treatment of cancer has been of recent interest[17]. However cancer prevention using natural products has become a new trend in cancer control, as high consumption of certain natural products is believed to contribute to decreased risks of several types of cancer[18,19]. Previous studies have shown that some specific herbs and natural compounds derived from potential herbs were able to effectively interfere with tumor progression, inhibit angiogenesis and block metastasis[20].In this study using two human cancer cell lines from colon and gastric cancers with different proliferative potentials, we analyzed the effect of methanolic extracts of *P. lentiscus* on cell viability, morphology, cell cycle and the induction of apoptosis.

A lot of work has focused on the effect of *P. lentiscus* (resin, chios mastic gum (CMG)) in the treatment of cellular cancer, and more specifically on growth inhibition, involving apoptosis and the cell cycle. However, few studies have been conducted on the Tunisian variety of *P. lentiscus*, therefore the objective of our study was to look specifically at the effect of fruit and the stem extracts on the gastric cancer AGS and colon cancer CaCo2 cell lines.

Based on the results obtained, we found that *P. lentiscus* extracts inhibited cell viability in AGS and CaCo2. The toxic effects of plant extracts resulted in a decrease in mitochondrial dehydrogenase. Whole aerial parts of the plant inhibited mitochondrial respiration with a percentage ranging from a party. The extract caused a significant concentration-dependent inhibition of mitochondrial respiration at varying concentrations (400, 40 and 4 µg/ml) in the two cell lines.

We also found that, in both cell lines, the cytotoxic effect of leaf and fruit extracts were close to 50% at 400 µg/ml. In contrast, the doses inducing 50% cell growth inhibition of the stem extracts were greater than 400 µg/ml in both cell lines. Furthermore, the best anti-proliferative effects in both cell lines were noted with leaf extracts. Similar study was published recently, which showed that an alcoholic extract from deterpenated *P. lentiscus* leaves induced the highest cell viability inhibition against C6, SH-SY5Y, and SK-N-BE - (2)-C cell lines[21].

Apoptosis plays a crucial role in eliminating the mutated neoplastic and hyperproliferating neoplastic cells from the system and therefore it is considered as a protective mechanism against cancer progression[22]. Apoptosis is a well known mechanism of programmed cell death that does not injure normal neighboring cells and avoids local inflammation[23].

By analyzing PI incorporation with flow cytometry and DAPI fluorescent staining by microscopy (Figures 3 and 4), we found that methanolic extracts of leaves, fruit and stems induced apoptosis in both cell lines at a concentration of 400 µg/ml. Furthermore, if we compare the leaf, fruit and stem extracts, it is apparent that leaf extracts are more potent than the other two, with a higher level of induction of apoptosis (of over 50% for both cell lines).

This significantly increased percentage of apoptotic cells suggests a blockade in the cell cycle progression leading to a programmed cell death. Moreover, the activation of apoptosis is believed to play a critical role in both the chemoprevention and treatment of human carcinomas[24].

The morphological changes of the cells after treatment with methanolic extracts of *P. lentiscus* were examined by phase contrast microscopy. Morphological changes such as membrane blebbing, chromatin condensation and the presence of apoptotic bodies were observed in treated AGS and CaCo2 cells, confirming an apoptotic phenotype (Figure 4). These results suggest that *P. lentiscus* extracts can induce apoptotic cell death in both colon cancer and gastric cancer cell lines.

Our results are in agreement with results obtained in previous publications using other cell lines. In 2007, Balan et al.[25] demonstrated that a 50% ethanol extract of the plant-derived product, CMG, contains compounds which inhibit proliferation and induce cell death of HCT116 human colon cancer cells *in vitro*.

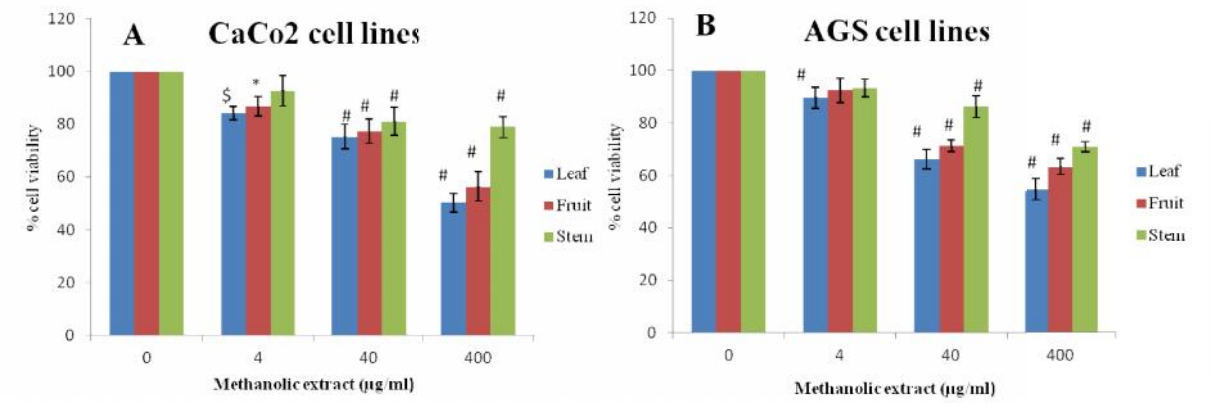

Figure 1

Effect of methanolic extracts of *Pistacia lentiscus* on cell viability of human colon and gastric cancer cell lines.

Cell viability was determined using the MTT assay 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay on CaCo2 **(A)** and AGS **(B)** cells, as described in Materials and Methods . Each bar represents the mean ± SE values of three separate experiments. * p<0.05, $ p< 0.01, # p<0.001 compared to the control.

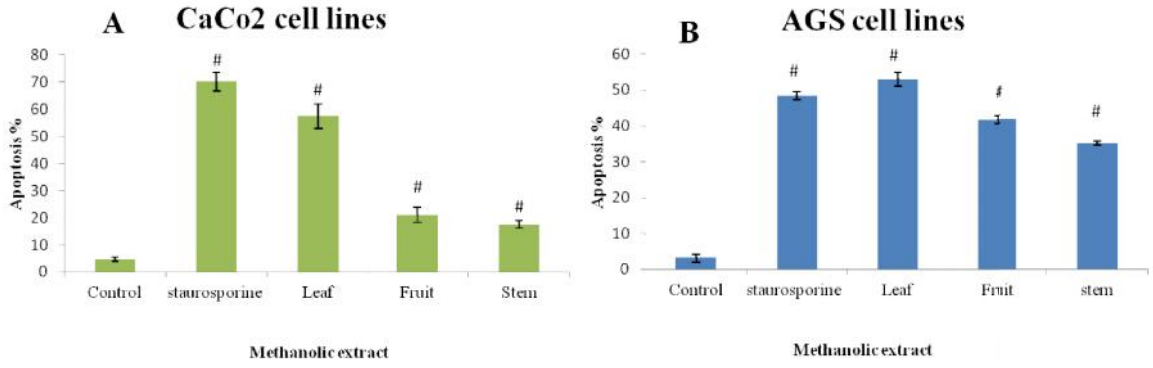

Figure 2

***P. lentiscus* extracts induce apoptotic cell death in a panel of human colon and gastric cancer cell lines**.

The percentage of apoptotic cells was determined by PI incorporation and flow cytometry analysis as described in Material and Methods, after treatment of CaCo2 (A) and AGS (B) cells with leaf, fruit and stem methanolic extracts or staurosporine (1 mM) . The data shown are the mean ± SD of three independent plates. # p<0.001 compared to the control.

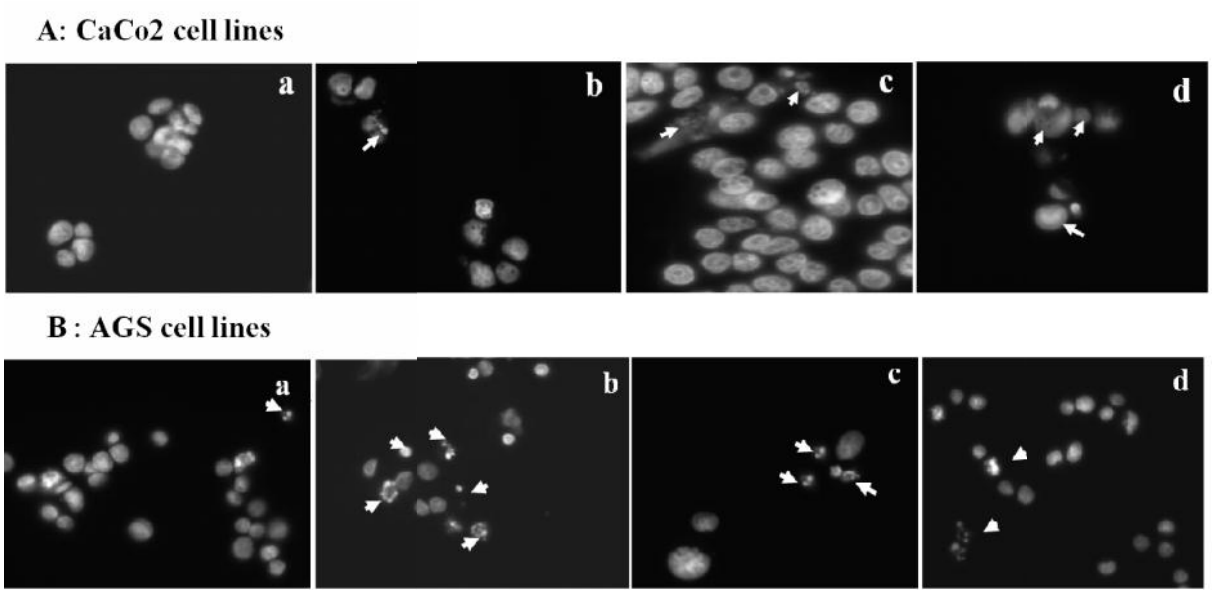

Figure 3

DAPI immunoflurescence shows apoptosis induction in CaCo2 and AGS cells

Apoptosis induction by methanolic extract in CaCo2 (A) and AGS (B) with a, b, c, d in control , leaf, fruit and stem respectively. This was analysed by immunofluorescence after nuclear staining with DAPI. Cells undergoing apoptosis and presenting chromatin condenstaion and nuclear fragmentation are indicated by arrows. (Magnification is 400×).

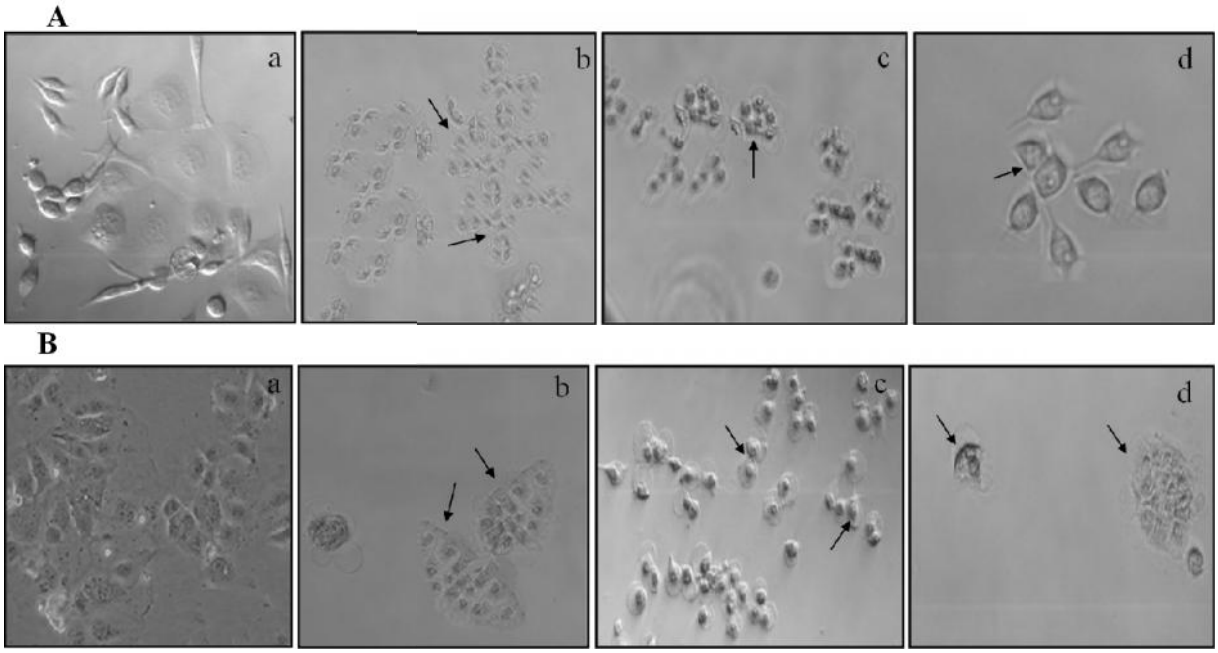

Figure 4

A,B: Morphologic changes of cells observed under a phase-contrast light microscope.

(Magnification is 400×). (**A**) AGS cells. a: Control group; b: Leaf extract ; c: Fruit extract; d: Stem extract. (**B**) CaCo2 cells. a: Control group; b: Leaf extract ; c: Fruit extract; d: Stem extract. The arrows indicate cell death characterized by cellular shrinkage the cells change form rounded and detached from the cell culture

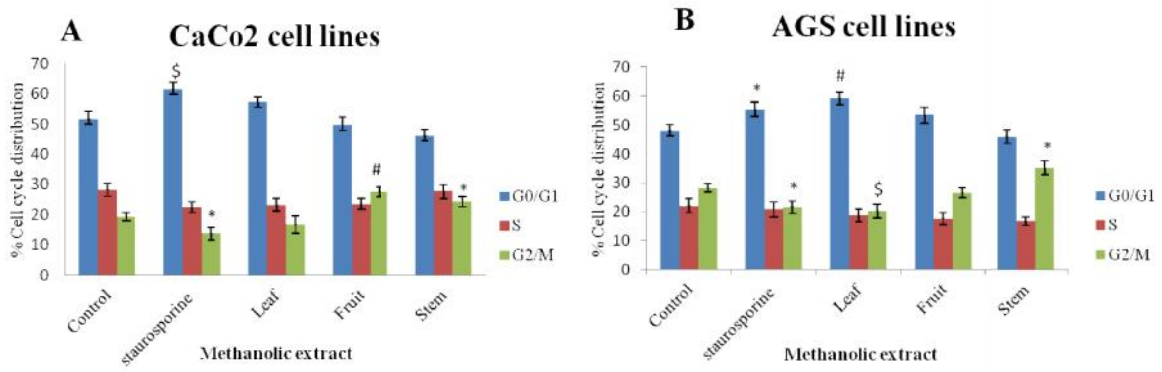

Figure 5

Effect of methanolic extracts of *Pistacia lentiscus* on cell cycle distribution in CaCo2 and AGS cell lines.

CaCo2 (A) and AGS (B) cells were cultured in the presence of 400 µg/ml of leaf, fruit, stem extracts of *P. lentiscus* or staurosporine at 1 mM for 24 h. Then the cells were collected, stained with PI and their DNA content was determined by flow cytometry analysis. The percentage of cells in the different phases of the cell cycle was calculated. Data shown are means ± SD from triplicate determinations. The level of significance for the different cell populations after *P. lentiscus* extract treatment was calculated by ANOVA with respect to the corresponding controls in the absence of extracts. The change in cell numbers in the different phases are significant at * $p<0.05$, $ $p< 0.01$ and # $p<0.001$ compared with the control.

According to the research done on the phytochemical the mastic gum inhibits the proliferation of LNCaP cells (Androgen responsive human prostate cancer cell line) by androgen via the androgen receptor (AR) which was used to treat prostate cancer[26]. Another study proved that CMG extract acts as a potential anti-tumor agent in the human oral squamous carcinoma YD-10B cell lines[27].

The magnitude of apoptosis in CaCo2 and AGS cells, induced by the extracts of the different parts of the *P. lentiscus* plant at a concentration of 400 µg/ml, is in agreement with the resting cell viability measured, suggesting a blockade along the cell cycle at different phases. At this concentration of leaf extract, the decreased viability was similar to the increase in cell death by apoptosis for both cell lines, suggesting a similar effect on cell cycle.

Flow cytometry analyses of the DNA content of *P. lentiscus* extract treated AGS cells showed that the leaf and fruit extracts caused a G_0/G_1 phase arrest with a marked decrease of cells in the S and G_2/M phases. Incidentally, the stem extracts induced a marginal increase in the G_2/M phase. Furthermore, treatment of CaCo2 cells with fruit and stem extracts for 24 h led to the arrest of cells in the G_2/M phase at the 400µg/ml concentration. However, a treatment with leaf extracts caused a significant arrest of the cells in the G_0/G_1 phase. Based on the previous findings and the present results we conclude that the methanolic extracts of *P. lentiscus* induce a strong cell cycle arrest in both cell lines.

Differences in the profiles of biological activities of the various extracts might show the presence of different constituents especially between the leaves, fruit and stems because strong anti-proliferative and proapoptotic effects were noted with the leaf extract.

Pistacia species can be used as an important source of natural antioxidants. They contain phenolics and flavonoids such as quercetin and α-tocopherol[28] .Phenolics affect cancer cells via cell cycle arrest and activation of apoptotic signal transduction pathways[29]. Cell growth, cell cycle related pathways and apoptosis have been known as targets of anthocyanin in vitro and in vivo in colon, hepatic and leukemia cells[30].

According to the literature, Balan et al[31] showed that a Chios mastic gum (CMG), induce death of HCT116 human colon cancer cells in vitro via the process of anoikis. Consequently, Hexane extract of CMG exerts its inhibitory effect on proliferation via induction of cell cycle arrest at the G1 phase detachment of the cells from the substrate and subsequent apoptosis.

In the present study we have proven, as mentioned in literature, that the extract of the leaves causes an accumulation of cells in the G_0/G_1 phase in colon cancer as well as in gastric cancer cells. We also showed that the extract of the fruit has the same effect as that of the leaves on CaCo2 cells. On AGS cells, the fruit and the stem extracts have weaker effects since the result is the increase of cells in the G_2/M phase compared with the untreated cells.

In conclusion, leaf extracts of *Pistacia lentiscus* were the most effective inhibitors of cell viability, apoptosis and cell cycle alterations because they presented a cytotoxic effect close to 50%, a high

induction of apoptosis and they induced a G_0/G_1- cell cycle arrest in both cell lines when compared to the other extracts. Finally, *P. lentiscus* leaf extract can first be used as a botanical drug in cancer patients, before it can be thoroughly analyzed for the isolation, characterization and testing of its anti-cancer active compounds.

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