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Original Article

In vitro Assessments of Cytotoxic and Cytostatic Effects of *Asparagus aphyllus*, *Crataegus aronia*, and *Ephedra alata* in Monocultures and Co-Cultures of Hepg2 and THP-1-Derived Macrophages

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ABSTRACT

Introduction: Herbal-based medicines are widely used for the prevention and treatments of diverse diseases especially in growing countries as well as many developed countries. Although some of herbal-based medicines have promising therapeutic properties, many of them remain untested and their safety and efficacy were not scientifically assessed. Based on knowledge from traditional Greco-Arab herbal medicine, this *in vitro* study aims to evaluate cytotoxic and cytostatic effects of three traditionally used anti-diabetic and anti-cancer medicinal plants. Out of the traditional medicinal plants, *Asparagus aphyllus*, *Crataegus aronia*, and *Ephedra alata* are widely used in many Mediterranean countries as natural remedies. **Methods:** Human THP-1- derived macrophages, HepG2 cells and their co-cultures were used in this *in vitro* study. Cells were treated for 24h (cytotoxic effects) and 72h (cytostatic effects) with increasing concentrations (0-1000 μg/ml) of water/ethanol extracts from *Asparagus aphyllous* (AA-extract), *Crataegus aronia* (CA-extract) and *Ephedra alata* (EA-extract). Cytotoxic and cytostatic effects were assessed using MTT assay and LDH assay. **Results:** No significant cytotoxic effects were seen with the three extracts up to concentration of 500 μg/ml. A slight cytotoxic effect was observed with CA-extractin HepG2 monocultures at concentrations higher than 500 μg/ml. Significant cytostatic effects were measured with CA-extract and EA-extract in monocultures and co-cultures. The cytostatic activity of the extracts was more potent in co-cultures reaching IC50 of 178 μg/mL and 380 μg/mL for CA-extract and EA-extract, respectively. **Conclusion:** These results indicate that the traditionally known anti-cancer effects of CA-extract and EA-extract might be mediated in part through cytostatic effects.

Key words: *Asparagus aphyllus*, *Crataegus aronia*, Cytostatic, Cytotoxic, *Ephedra alata.*

INTRODUCTION

Herbal-based remedies are applied for the prevention and treatments of diverse diseases especially in growing countries as well as many developed countries.[1] The number of people using medicinal plants is rapidly increasing and therefore national health authorities are beginning to express concern over the safety and efficacy of these products since almost all products are sold over the counter and are not registered.[2,3] Although some of herbal-based medicines have promising therapeutic potential, many of them remain untested and their safety and efficacy were not scientifically assessed. The safety of most herbal products is compromised by inadequate labeling, lack of suitable quality controls and the absence of appropriate patient information.[4] Regrettably, consumers

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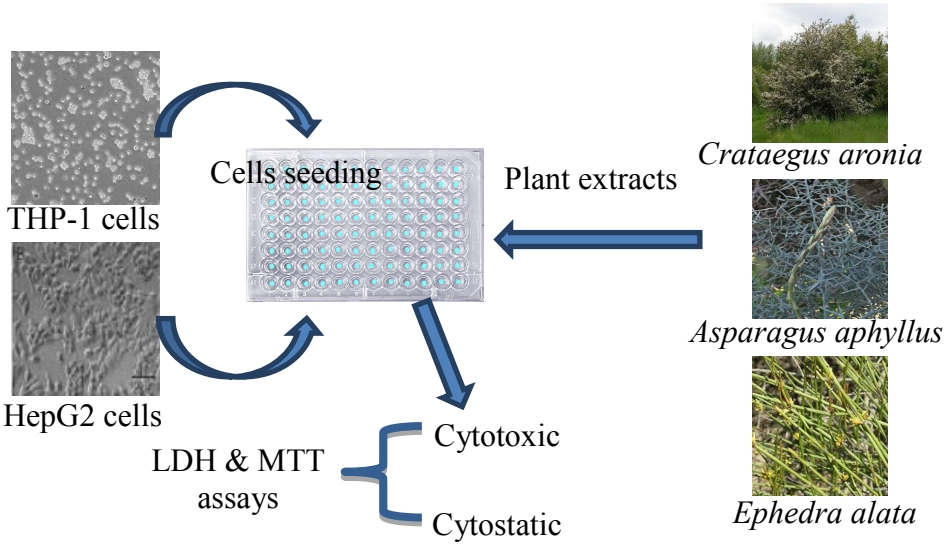

Graphical Abstract

refer to medicinal plants as safe products since they are 'natural' and use them as home remedies without regulations or prescription in most cases. The paradigm that herbal remedies or drugs are safe and without side effects is untrue and misleading. Some herbs have been shown to be poisoning, cause serious injuries, life-threatening conditions, and even death.[1,5]

Herbal-induced cytostatic and cytotoxic effects can be therapeutically effective when directed to attack tumorigenesis proliferations. For instance, *Peganum harmala* seeds extracts were shown to have both cytotoxicity and antitumor activity in several tumor cell lines *in vitro* and *in vivo*.[6,7] The cytotoxic activity of diverse anticancer agents and herbal extracts are usually based on DNA damage and the subsequent induction of apoptosis. Beside cytotoxic reactions, cancer cells proliferation can be prevented by cell cycle block or delay.[8] Cytostatic drugs, such as epirubicin and cyclophosphamide (stop cancerous cell proliferation), and cisplatin (toxic to tumor liver cells by stopping cell proliferation *in vitro*) are widely used to treat cancer.[9]

Of the traditional medicinal plants, *Asparagus aphyllus* (AA), *Crataegus aronia* (CA), and *Ephedra alata* (EA) are widely used in Palestine as well as in many Mediterranean countries as natural remedies.[1] *Ephedra* species are traditionally usedto diminish obesity, decrease the blood glucose and to control blood pressure as diuretic.[10] CA is traditionally used to treat cardiovascular diseases, and digestive system imbalances. Various Crataegus species are used as a remedy for cancer, diabetes, and heart problems.[11] Other therapeutic mechanism include anti-oxidant, antimicrobial activity, anti-inflammation, and antidiuretic activity.[12-14]

EA species are used to treat gut troubles and are known to diminish obesity.[15] Various *in vivo* and *in vitro* studies showed that different species of Asparagus exhibit anti-inflammatory effects,[16] anti-microbial activity,[17] reduce writhing and pain,[18] and lower glucose and cholesterol levels in the blood.[19-22]

Here in we tested AA, CA and EA toxicity *in vitro* on hepatocytes cell line (HepG2) and THP-1 macrophages derived cells in mono and co-culture. Their cytostatic effect is also discussed.

MATERIALS AND METHODS

Preparation of plant extracts: The plants were collected from the hills of the West Bank during the spring and summer after their identification by qualified botanists. The plants were dried for 7–10 days in the shade at environmental temperatures. The dried plants were then ground and stored in cloth bags at 5ºC until their transfer to the laboratory for preparation of the plant extracts. Fifteengrams of the hand grinded plant material were added to 100 ml of 50% ethanol (Yavin Yeda, Israel) in double distilled water and boiled for 10 minutes under stirring. Extract supernatants obtained were passed through a 0.2 mm filter, and freeze dried. The powders were stored at-70°C for further evaluation.

Cells: The human monocytic cell line THP-1 (ATCC 202-TIB) and human hepatoblastoma cell line HepG2 (ATCC HB8065) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells from

the HepG2 cell line are known to retain differentiated parenchymal functions of normal hepatocytes, including the expression of P450 isoenzymes thus permit long-term studies to be performed.

Mono-cultures: THP-1 cells and HepG2 cells were grown in RPMI-1640 and DMEM-5671 medium, respectively with a high glucose content (4.5 g/l), supplemented with 10% vol/vol inactivated fetal calf serum (FCS), 1% nonessential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 μg/ml streptomycin (Sigma-Aldrich). For the cytotoxic assays, 20,000 cells/100 μL medium from HepG2 or THP-1 cell lines were seeded in 96-microtiter plates. THP-1 cells were then differentiated to macrophages by the addition of PMA (100 ng/ml) and Vitamin D3 (0.1 μM) for 24h. For cytostatic assays, 5,000 cells/100 μL medium from HepG2 or THP-1 cell lines were seeded in96-microtiter plates. THP-1 cells were then differentiated to macrophagesas described before.

Co-cultures: As for the cytotoxic and cytostatic assays in monoculture system, 10,000 (cytotoxic) and 2500 cells (cytostatic) from THP-1cell line were seeded in 100 μL RPMI media per well of 96-microtiter plates. Cells were then differentiated to macrophages by the addition of PMA (100 ng/ml) and Vitamin D3 (0.1 μM) for 24 h. After removal of RPMI media, 10,000 (cytotoxic) and 2500 (cytostatic) cells from HepG2 cell line were seeded in 100 μL DMEM media per well of 96-microtiter plates.

Cytotoxic and cytostatic effects in monoculture system

MTT assay: The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of the cells. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells as described in.[6] Twenty-four hours after cell seeding, cells from both cell lines were incubated with increasing concentrations of water/ethanol extracts of *Asparagus aphyllus (*AA-extract), *Crataegus aronia* (CA-extract), and *Ephedra alata* (EA-extract)(0-1000 μg/mL) for 24 hours in cytotoxic test and for 72 hours in cytostatic test at 37°C. Following the removal of the media from each well, cells were washedin phosphate buffered saline. The cells were then incubated in serum-free RPMI to which MTT (0.5 mg/mL) was added to each well (100 μL), and incubated for a further four hours in the dark. Then the medium was removed, washed, and the cells were incubated for 15 minutes with 100 μL of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals occluded in the mitochondria. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Lactate dehydrogenase: In the Lactate dehydrogenase (LDH) assay the leakage of the cytoplasm located enzyme LDH into the extra cellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium was indicative of cell membrane damage.[6]

Twenty-four hours after cell seeding and differentiation of THP-1 cells, cells were exposed to varying concentrations of the plant extracts extract (0-1000 μg/mL). After 24 hours in cytotoxic test and for 72 hours in cytostatic treatment, the supernatants were collected from each well and LDH activity was tested. Cell monolayers were then treated with a cell lysis solution for 30 minutes at room temperature. LDH activity was measured by using Cyto-Tox 96 kit (Promega, WI, USA) in accordance with the manufacturer's instruction. The absorbance was determined at 490 nm with 96-well plate ELISA reader. The percent of LDH release from the cells was determined using the formula:

$$LDH\ release = \frac{Absorbance\ of\ the\ supernatant}{absorbance\ of\ the\ supernatant\ and\ cell\ lysate} \times 100$$

Cytotoxic and cytostatic effects in co-culture system

As for the cytotoxic and cytostatic assays in monoculture system, 10,000 and 2500 cells from THP-1cell line were seeded in 100 μL RPMI media per well of 96-microtiter plates, respectively. Cells were then differentiated to macrophages by the addition of PMA (100 ng/ml) and Vitamin D3 (0.1 μM) for 24 h. After removal of RPMI media, 10,000 (cytotoxic) and 2500 (cytostatic) cells from HepG2 cell line were seeded in 100 μL DMEM media per well of 96-microtiter plates. Twenty four hours later cells were incubated with increasing concentrations of AA-extract, CA-extract, and EA-extract (0-1000 μg/mL) for 24 hours at 37°C. MTT assay and LDH assay were then carried out as described for themonoculture system above.

Statistical analysis

Error limits cited and error bars plotted represent simple standard deviations of the mean. When comparing different samples, results were considered to be statistically significant when $P<0.05$ (Student's t test for unpaired samples).

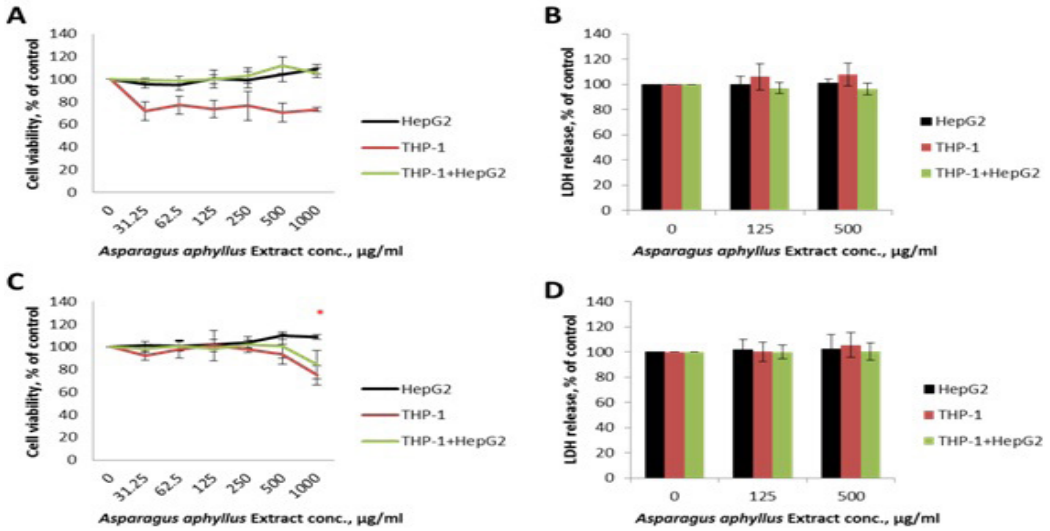

Figure 1: MTT and LDH Assay in THP-1-derived macrophages, HepG2 cells and their co-cultures after 24h (cytotoxic effects) and 72h (cytostatic effects) treatment with increased concentrations of extract from *Asparagus aphyllus*. The absorbance of the MTT formazan (A and C) was determined at 570 nm in an ELISA reader. LDH activity (B and D) was determined at 490 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of *Asparagus aphyllus* treated cells relative to untreated cells. 125 and 500 µg/ml extract concentrations correspond to 2.1 and 2.7 in a logarithmic value respectively. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

RESULTS AND DISCUSSION

In vitro culture models that employ human liver cells represent potent tools for predictive studies on drug metabolism and toxicity. In general, cells isolated from various tissues or cell lines are applied. *In vitro* methods have the advantage of relatively well-controlled variables and are generally accepted as a very effective method for toxicity testing. However, the fact that cells and tissues *in vivo* do not exist in isolation, but communicate with and are interdependent on neighboring tissue makes it essential to simulate the *in vivo* situation, where for example, the microenvironment of the hepatocytes within the liver acinus involves gradients in hormones, extra cellular matrix components, nonparenchymal cells and effective exposure levels of xenobiotics from the periportal to the pericentral compartment. Conventional homotypic hepatocyte cultures do not include the possible contribution of nonparenchymal liver cells, particularly Kupffer cells, to the pharmacological and toxicological consequences after exposure to xenobiotics. Therefore, in the present study we evaluated the cytotoxic and cytostatic effects of three plant extracts using co-cultures of cells from the human hepatocyte cell line (HepG2) and cells from the human monocyte cell line (THP1). In this co-culture system both cell types have direct cell-to-cell contacts and are maintained in more "*in vivo* like" culture conditions than in the monoculture system.

Based on knowledge from traditional Greco-Arab herbal medicine, this *in vitro* study aims to evaluate the cytotoxic and cytostatic effects of three traditionally used anti-diabetic and anti-cancer medicinal plants. Cytotoxic and cytostatic effects of water/ethanol extracts from *Asparagus aphyllus* (AA-extract), *Crataegus aronia* (CA-extract) and *Ephedra alata* (EA-extract) were evaluated here in cells from the hepatocyte cell line (HepG2) and cells from the monocyte cell line (THP-1) and their co-cultures. MTT assay LDH assay were carried out 24 h (cytotoxic effects) and 72 h (cytostatic effects) after treatment with increasing concentrations (0-1000 µg/mL of culture medium) of extracts from the three plant extracts.

Activated hepatic macrophages (Kupffer cells) play an essential role in liver injury as well as in xenobiotic metabolism.[23,24] Following binding of LPS to the cluster of differentiation (CD) 14 protein, the complex triggers a signal cascade involving the nuclear factor kappa B. This factor enhances the expression of inflammation-related genes. The acute-phase response is regulated by cytokines released by activated Kupffer cells, notably interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α).[25] Among these cytokines, IL-6, also known as hepatocyte-stimulating factor, is a major inducer of the acute phase response. IL-6 is produced not only by Kupffer cells but also other cell types including monocytes/macrophages, and hepatocytes. Hepatocytes produce also nitric oxide (NO) during chronic hepatic

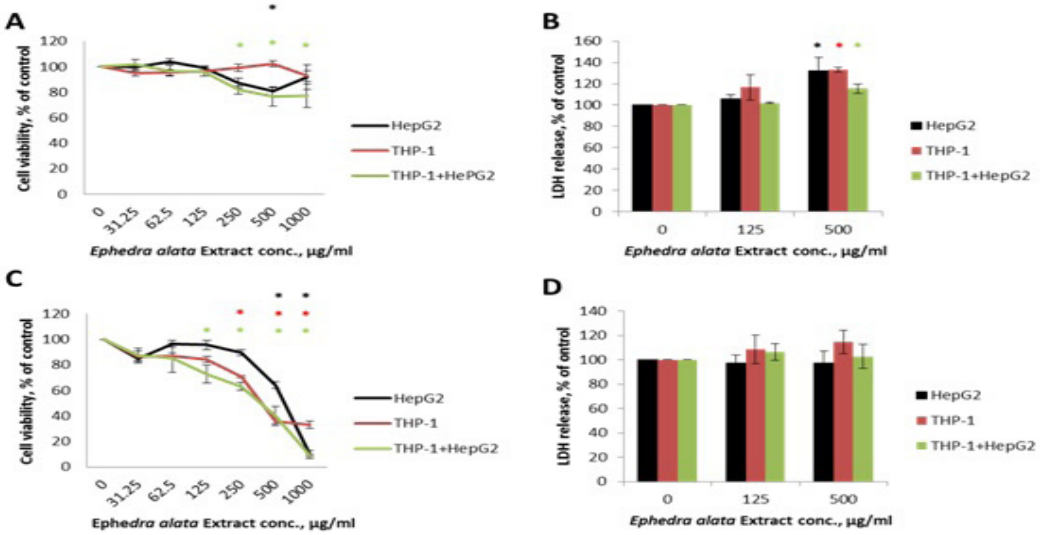

Figure 2: MTT and LDH Assay in THP-1-derived macrophages, HepG2 cells and their co-cultures after 24h (cytotoxic effects) and 72h (cytostatic effects) treatment with increased concentrations of extract from *Ephedra alata*. The absorbance of the MTT formazan (A and C) was determined at 570 nm in an ELISA reader. LDH activity values (B and D) were determined at 490 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of *Ephedra alata* treated cells relative to untreated cells. 125 and 500 µg/ml extract concentrations correspond to 2.1 and 2.7 in a logarithmic value respectively. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

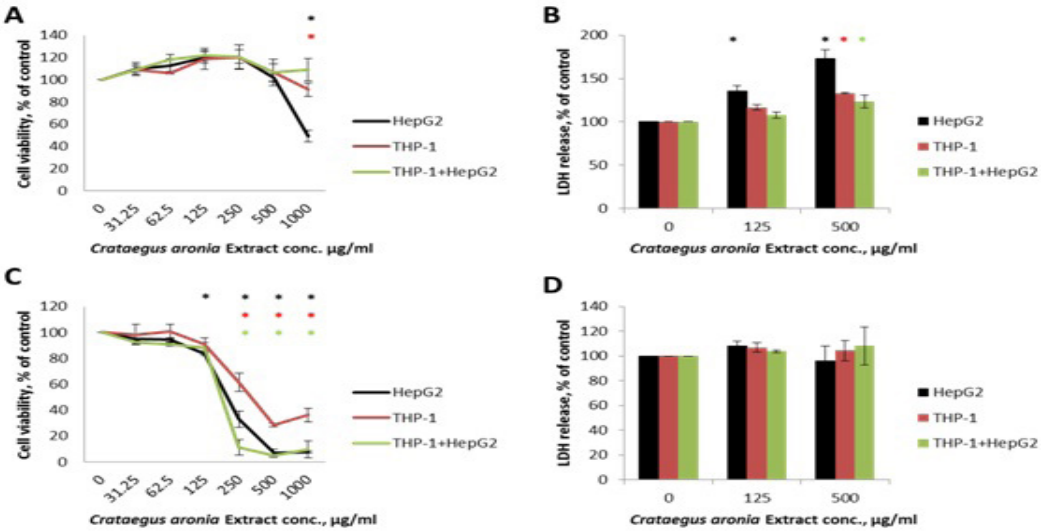

Figure 3: MTT and LDH Assay in THP-1-derived macrophages, HepG2 cells and their co-cultures after 24h (cytotoxic effects) and 72 h (cytostatic effects) treatment with increased concentrations of extract from *Crataegus aronia*. The absorbance of the MTT formazan (A and C) was determined at 570 nm in an ELISA reader. LDH activity (B and D) was determined at 490 nm in an ELISA reader Cell viability was defined as the absorbance ratio (expressed as a percentage) of *Crataegus aronia* treated cells relative to untreated cells. 125 and 500 µg/ml extract concentrations correspond to 2.1 and 2.7 in logarithmic values respectively. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

Table 1: IC50 values (µg/mL) of the plant extracts were measured in three culture systems after 72 h at cell density of 5000 cell/well using the MTT test

Extract	THP-1 cells	HepG2 cells	HepG2 cells + THP-1 cells
AA-extract	Over 1000	Over 1000	Over 1000
EA-extract	372	617	380
CA-extract	302	389	178

inflammation[26] and *in vitro* in response to conditioned medium from activated Kupffer cells or to a mixture of LPS and TNF-α, IL-1 and IFN-μ. In the liver, TNF-α production is not restricted to Kupffer cells.[27,28] LPS affects the acute phase response via hepatocyte-derived IL-6 and TNF-α in an autocrine loop and the NO production of parenchymal liver cells showed.[28] TNF-α is also involved in inducing cell damage by promoting oxidative stress in mitochondria that occurs as a result of an imbalance between oxidants and antioxidants in favor of oxidants.[29] TNF-α stimulates the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS have been implicated in the pathogenesis of many forms of liver disease. When liver cells are exposed to excess of ROS, oxidative stress occurs and affects many cellular functions. However, cells are equipped with antioxidant systems. Liver macrophages (Kupffer cells) play also an important role in the modulation of drug metabolism in liver and its therapeutic and toxicological effects.[30] Therefore, we used here co-cultures of human hepatoblastoma cell line HepG2 cells and monocyte cell line THP-1. THP-1 cells are widely used to test anti-inflammatory effects *in vitro*.[6,31] These cells differentiate to macrophages by the addition of PMA (100 ng/ml) and Vitamin D3 (0.1 μM) for 24 h. The co-cultures of hepatoblastoma and THP-1 derived macrophages were used in order toenable cultured cells to grow at higher cell density and to maintain more *in vivo* like cell-to-cell interactions. Thus to obtain more reliable results *in vitro*. The HepG2 cell line retains differentiated parenchymal functions of normal hepatocytes and can be grown indefinitely, thus permitting long-term studies to be performed.[32] After treatment of HepG2 cells and co-cultures of HepG2 cells and THP-1 derived macrophages with various concentrations of AA-extract, CA-extract, and EA-extract for 24 h and 72 h, the cytotoxic and cytostatic effects were evaluated, by MTT test and the LDH assay. The MTT test is widely used in the *in vitro* evaluation of plant extracts and purified drugs biosafety.[33] It is designed to be used for the quantification of both cell proliferation and cell viability by measuring the metabolic activity of a mitochondrial enzyme succinate dehydrogenase.

HepG2 cells and co-cultures of HepG2 and THP-1 were exposed to increasing concentrations (0-1000 μg/mL) of the three plants extracts for 24 h and 72 h. Following the removal of the plant extracts from each well, cells were washed in phosphate buffered saline, and the MTT assay was carried out as described in the methods section. Unattached HepG2 cells and THP-1 cells (grow in suspension) are removed from the co-cultures during the washing process. In order to distinguish between cytotoxic and cytostatic effects we applied the Lactate dehydrogenase (LDH) assay. Lactate dehydrogenase, an enzyme located in the cytoplasm, catalysis the conversion of lactate and pyruvate. Cellular death or a 'leak' in a cell membrane is the two possible causes increasing lactate dehydrogenase activity in the culture media. In the case of cytotoxic effects, the LDH values elevated and the MTT values are decreased. In contrast, cytostatic effects are found when the MTT values are decreased and LDH values remain unchanged (compared to untreated control cells). Constant LDH value (treated cells vs. control) indicates that the cells are alive but do not proliferate.

AA-extracts exhibited no sign of any cytotoxic or cytostatic effects at all concentrations tested in all three-culture systems as measured with MTT test (Figure 1A, 1C) and LDH assay (Figure 1B, 1D. EA-extracts showed clear and significant cytostatic effects (Figure 2C, 2D) at non-cytotoxic concentrations. A dose-dependent decrease in MTT was apparentafter treatment with EA-extract for 72h (Figure 2C). No significant change was seen in the LDH values (Figure 2D). No sign of cytotoxic effects were seen at all concentrations tested as measured with MTT assay (Figure 2A) and LDH assay (Figure 2B). These results indicate that EA-extract induces cytostatic effects in all three cell culture systems. A slight, but significant cytotoxic effects were observed in HepG2 monoculture after treatment with CA-extracts at concentrations higher than 250 μg/mL as measured with MTT (Figure 3A) and LDH assay (Figure 3B). This extract showed a significant and dose dependent cytostatic effects at concentrations higher than 250 μg/mL (Figure 3C, 3D).

The IC50 values of the cytostatic effects of EA-extract and CA-extracts shows that CA-extracts affect the cells at much lower concentrations than EA-extract. The extracts' cytostatic efficiency was higher in the co-culture system than in the monoculture system. The IC50 values for CA-extracts in the co-cultures were about 55% and 42% lower than in the HepG2 and THP-1 monocultures, respectively. Results of IC50 values with the EA-extract in co-culture were lower by 40% than in the HepG2 monocultures while remains similar to THP-1 monocultures (Table 1).

The observed difference in the cytostatic effects EA-extract and CA-extract in HepG2 and THP-1-derived macrophages and in their co-cultures may be the results of cell-to-cell interactions and/or of cytokines produced by THP-1-derived macrophages. Hepatocyte mono-cultures do not include the possible contribution of non-parenchymal liver cells, particularly Kupffer cells, to the

pharmacological and toxicological consequences after exposure to xenobiotics.[32]

Taken together, it seems that the observed higher cytostatic effects in the co-culture are the results of monocyte-derived factors produced after activation of these cells by extract from EA-extract and CA-extract.

CONCLUSION

Based on knowledge from traditional Greco-Arab herbal medicine, this *in vitro* study aims to evaluate cytotoxic and cytostatic effects of three traditionally used anti-diabetic and anti-cancer medicinal plants in human THP-1-derived macrophages, HepG2 cells and their co-cultures using MTT assay and LDH assay. In conclusion, it seems that the observed higher cytostatic effects in the co-culture are the results of monocyte-derived factors produced after activation of these cells by extract from EA-extract and CA-extract.

ACKNOWLEDGMENTS

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Highlights of Paper

- Traditionally known anti-cancer effects of, *Crataegus aronia*-extract and E*phedra alata*-extract might be mediated in part through their cytostatic effects.
- *Asparagus aphyllus* (AA), *Crataegus aronia* (CA), and *Ephedra alata* (EA) water/ethanol extracts were not toxic for HepG2 and THP1 cells up to 500 μg/ml.
- The cytostatic IC50 for CA-extract and EA-extract was 178 μg/mL and 380 μg/mL, respectively.

Author Profile

- Professor (PhD) Badiaâ Lyoussi is the head of laboratory physiology–pharmacology & environmental health at University Sidi Mohamed Ben Abdullah, Fez, Morocco. Prof. Lyoussi had more than 80 scientific publications and chapters in books in the area of acute and chronic toxicity of medicinal plants used in traditional medicine, ethnopharmacology and management of several pathologies like diabetes, obesity, hypertension, and metabolic syndrome. She is membermany scientific organizations around the world. She is the president of the Arab women Scientific foundation for North Africa.

- Mr. Abdalsalam Kmail is a PhD student at Fez. University, Morocco. He got his MSc. in Engineering Biotechnology at Sabanci University, Turkey. Mr. Kmail is working as a lecturer at the Arab American University –Jenin. He is interested in the medicinal plants and their effects on diabetes and inflammation. He has five publications and several presentations and posters in this field.

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