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Original Article

In vitro evaluation of anti-inflammatory and antioxidant effects of *Asparagus aphyllus* L., *Crataegus azarolus* L., and *Ephedra alata* Decne.in monocultures and co-cultures of HepG2 and THP-1-derived macrophages

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ABSTRACT

Introduction: *Asparagus aphyllus* L.,*Crataegus azarolus* L., and *Ephedra alata* Decne.have been used in traditional Greco-Arab herbal medicine to treat various metabolic and inflammatory diseases. However, to our knowledge, no scientific evidences regarding their anti-inflammatory effects have been published yet.This study aimedto evaluate the antioxidant and anti-inflammatory properties of *Asparagus aphyllus* L., *Crataegus azarolus* L., and *Ephedra alata* Decne.extracts using *in vitro* mono and co-culture systems. **Methods:** In the first phase of the current study we measured the total phenol, flavones and flavonol contents as well as the antioxidant activity using the DPPH scavenging assay, ABTS assay, and reducing power of the three plant extracts. In second phase, we used THP-1-derived macrophages as a monoculture and co-culture with hepatic cell line (HepG2) to assess the effects of the three plant extracts on the production of LPS-induced pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines.**Results:** All three extracts exhibit relatively high levels of phenolics, flavones and flavonols, which correlate with their antioxidant capacities. All three extracts were found to modulate the secretion levels of TNF-α, IL-6 and IL-10 at non-toxic concentrations as measured with MTT and LDH assays. **Conclusion:** Taken together, our results suggest that the traditional uses of these extracts as anti-inflammatory remedies may be mediated by their significant antioxidant capacity, inhibition of pro-inflammatory cytokines secretion concomitant with enhancement of anti-inflammatory cytokines secretion.

Key words: Anti-inflammatory, Antioxidant; *Asparagus aphyllus* L.; *Crataegus azarolus* L.;*Ephedra alata* Decne.

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INTRODUCTION

Inflammation is the first response of the immune system to infection, irritation, as well as to various other diseases such as cancer, cardiovascular diseases, obesity, hypertension, diabetes, autoimmune, and neurodegenerative disorders.[1] it involves directed migration and activation of leukocytes (neutrophils, monocytes and eosinophils) to the site of damage. Inflammation is linked to oxidant/antioxidant imbalance (an elevation in the reactive oxygen speciesin parallel to low levels of antioxidants). High levels of inflammatory markers and low level of antioxidants are observed in many inflammatory diseases[2,3] as well as in many pathological conditions such as malignancy, cardiovascular disease, diabetes type 2, kidney malfunction, gastrointestinal disorders, microbial infection, fibrogenesis, and neurological diseases. The imbalance between production of antioxidants versus getting rid of free radical species leads to oxidative stress.[4-6]

Inflammation is driven by the release of a wide range of pro-inflammatory mediators, such as the pro-inflammatory cytokines IL-1β, IL- TNF-α, IL- 6, IL 8, and IFN-γ. Anti-inflammatory cytokines that includes IL-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13 down regulate the inflammatory response. Chemo-therapeutic anti-inflammatory drugs including aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) contribute effectively in the inflammation and tumorigenic processes.[7-10]

Reactive oxygen and nitrogen species are free radical molecules that are generated within the body during metabolic reactions in cellsand can lead to oxidative stress as well as lipids, proteins, and DNA malfunction.[11,12] Reactive oxygen species are grouped into oxygen radicals (e.g., superoxide, hydroxyl radicals, and peroxyl radicals) and reactive nitrogen species (e.g., nitrogen dioxide, nitric oxide radicals and peroxynitrite). In general, antioxidantsare grouped into, primary antioxidants which function as scavenger for radical species, or secondary antioxidants which function as peroxide decomposers, metal chelators, oxidative enzyme inhibitors or UV radiation absorbers.[13] The body produces enzymes such as glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase, and produce natural antioxidants such as melatonin, thiol antioxidants, melatonin, specific metal proteins and coenzymes to reduceexcessive free radicals. Another source for the body antioxidants are from herbal-derived compounds, such as polyphenols, carotenoids, vitamin E, vitamin C, and trace elements. Various phytochemicals, such as polyphenols and flavonoids were found in both *in vitro* and *in vivo* studies to exhibit anti-inflammatory and antioxidant effects activities.[11,13]

Medicinal plants have attracted huge attention due to their diverse range of biological and therapeutic properties. Evidence has accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary, and alternative systems.[14-16] Of the traditional medicinal plants, *Asparagus aphyllus* L.(AA), *Ephedra alata* Decne. (EA),and*Crataegus azarolus* L.(CA) are widely used in many Mediterranean countries as natural remedies. *Asparagus aphyllus* L.(Prickly Asparagus in English, Halion in Arabic) is used in traditional Greco-Arab and Islamic medicine to treat various liver diseases liver diseases, diarrhea and dysentery.[14] Extracts of *Asparagus* species shown to inhibit the growth of *Candida albicans*, *Enterococcus faecalis* and *Staphylococcus*

aureus.[17] Compounds from the root of *Asparagus cochinchinensis* (dioscin and methylprotodioscin) were reported *in vitro* to inhibit the expression of MUC5AC mucin protein in the NCI-H292 epithelial cells, which partially validatestraditional usesin inhibiting hypersecretion in the mucus of pulmonary tracts.[18] Asparagus gum polysaccharide have immune-modulatory functions and were shown to accelerate apoptosis, diminishing tumor development of hepatocellular carcinoma in liver. Cladophyll and roots of *Asparagus in vivo* were reported to lower diastolic and diastolic blood pressure, left cardio-ankle vascular index score, total cholesterol level, and blood glucose.[19,20] *Ephedra alata* Decne. (Sand Camel's ephedra in English, O'leaq or Alanda in Arabic) is used in Greco-Arab traditional medicine to treat cancer, asthma and respiratory problems.[14] EA extracts are traditionally used (orally) in Palestine for the treatment of breast cancer patients.[21] The plant hascytostatic effects on different cancerous human cells.[22] It has been found that the ephedra extract has anti-pyretic and anti-asthmatic activity in Wistar rats and it decreased ulcer healing time in rats.[23] Antibacterial inhibition was also noted as an effect of EA on *B. subtilis*, *M. catarrhalis* and methicillin-resistant and non-resistant *S. aureus* bacteria.[24,25] Ephedra decreased NF-κB p65 activity leading to alleviation of lung damage and regulation of the cytokine production (IL-2 elevation and IL-1β, IFN-γ and TNF-α mRNA inhibition in lung tissues).[26,27]*Crataegus azarolus* L. (Azarolein English, Za'roor in Arabic), is used in the Greco-Arab traditional medicine to treat cardiovascular diseases, sexual weakness, cancer and diabetes.[14] *Crataegus* extracts were shown to be aneffective treatment for cardiovascular diseases such as, congestive heart failure, ischemic heart disease, arrhythmias and hypertension.[28] *Crataegus* species were used as a traditional medicine for the treatment of inflammation and gastro protection. They were also used as an antioxidant, hypolipidemic, and antimicrobial agent, as well as to stimulate digestion and improve blood circulation.[29,30] *Crataegus pinnatifida* can be used to treat asthma as it can treat allergic airway inflammation and can inhibit the expression ofintercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).[31] *Crataegus* extracts lowered the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum and inducedapoptosis in rats liver cells.[32] Fruits of *Crataegus* can be used to treat diabetes, as they were reported to inhibit the activity for α-glucosidase, protein-tyrosine phosphatase 1B (PTP1B), andadvanced glycation end products AGEs formation.[33] Recently, significant cytostatic effects have been measured with AA-extract and EA-extract in mono-cultures and co-cultures. The cytostatic activity of the extracts was more potent in co-cultures reaching IC_{50} of 178 μg/mL and 380 μg/mL for CA-extract and EA-extract, respectively.[22]

In this study, the anti-inflammatory, cytotoxicity as well and the anti-oxidative *in vitro* activity of *Asparagus aphyllus* L., *Ephedra alata* Decne. and *Crataegus azarolus* L. extracts were assessed in THP1 and HepG2 cell mono and co-cultures.

MATERIALS AND METHODS

Reagents and kits

Cell culture medium (DMEM and RPMI) and their supplements were purchased from (Biological industries, Israel).Reagents used in the antioxidant assays and MTT were purchased from (Sigma-Aldrich, USA). TNF-α, IL-6 and IL-10 kits were purchased from (R&D Systems, Inc., USA). LDH CytoTox 96 kits were purchased from (Promega, WI, USA).

Plants material

Plants of AA, CA and EA (Table 1) were collected from the hills of the West Bank during periods of April–July, 2015 after their identification by Dr. Nidal Jaradat, a qualified botanist at the Al-Najah University, Palestine. The samples were well air dried in the shade for 7-10 days. The dried plants were then ground to a powder and stored in cloth bags at 5^0C until their transfer to the laboratory for preparation of the plant extracts.

Plant extracts

Fifteen grams of air-dried powdered plants were dissolved in 50% water-ethanol solution under stirring for 10 min. at 70^0C. The extract supernatants obtained were passed through a 0.2 mm filter, and freeze dried. The powders were stored at-70^0C for further use. The net powder yield of the extracts/air dried plants were (AA: 270 mg/G, EA: 170 mg/G, CA: 156 mg/G).

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 and thus permit long-term studies to be performed.

Mono-cultures for assessing cytokines production

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). 250,000 cells/1 ml medium from HepG2 or THP-1 cell lines were seeded in 6-well plates. THP-1 cells were then differentiated to macrophages by the addition of PMA (100 ng/ml) and vitamin D3 (0.1 μM) for 24 h. The medium was then refreshed and stimulated with 1μg/mL LPS for further cytokines readings.

Co-cultures for assessing cytokines production

THP-1 cells were grown in RPMI-1640 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). 125,000 THP-1 cells/1 ml RPMI medium were seeded in 6-well 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. Cells then were washed three times and 125,000 HepG2 cells/ml DMEM medium were gown in the 6-well plates. The co-cultures were incubated for 24 h, then refreshed and stimulated with 1μg/mL LPS for further cytokines readings.

MTT assay

MTT viability assay was carried out as described by Kaadan *et al*.[34] Briefly, twenty-four hours after cell seeding and differentiation (in the case of THP-1 cells), cells were treated with increasing concentrationsof the plant extracts (0-250 μg/mL) for 24 hours at 37°C. The cells were washed and 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. The cells were washed and incubated for 15 minutes with 100 μL of acidic isopropanol (0.08 N HCl). The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader (BioRad, 680). Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

LDH assay

The lactate dehydrogenase (LDH) assay was carried out as described by Kaadan *et al*.[34] Briefly, twenty-four hours after cell seeding and differentiation (in the case of THP-1 cells) cells or for further 24 hours (in the coculture with HepG2 cells), were incubated withextracts with serial dilutions (0-250 μg/mL). After 24 hours the supernatants were collected and LDH activity was tested. Cell monolayers were then treated with

a cell lysis solution for 30 min at room temperature. LDH activity was measured by using CytoTox 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:

$$\frac{\text{The absorbance of the supernatant}}{\text{The absorbance of the supernatant and cell lysate}} \times 100$$

Quantification of TNF-α, IL-6, and IL-10 production, induced by LPS

THP-1 first were differentiated into macrophages in both mono and co-culture systems as outlined above in six well-plates. THP-1 (mono-culture system) and THP-1 with HepG2 (co-culture system) cells were washed three times and pretreated with a non-toxic concentration, 0 (negative control) or different concentrations (62.5, and 125 µg/mL) of the EA, CA or AA plant extracts., The cells were then treated with 1 µg/mL LPS. A positive control wascarried out usingLPS stimulated cells with no plant extract. The level of TNF-α, IL-6 and IL10 release were measured in the supernatants from stimulated cells by an ELISA kit (obtained from R&D Systems) after 4 h, 6 h, and 24 h of treatment, respectively according to the manufacturer's protocol. Plates were read at 450 nm on a multi scan bi-chromatic ELISA reader.

Total phenol content

The total polyphenol content was determined as described by Chang *et al.*[35] with slight modifications. Briefly, a volume of 0.1 ml of each extract was mixed with the 0.5 ml of Folin-Ciocalteu phenol reagent (1:10 dilution with distilled water) and 0.4 ml of aqueous 0.7 M Na_2CO_3 solution. The reaction mixture was incubated for 2 h and centrifuged at 1000 r/min. for 5 min. The absorbance was estimated in 1 ml cuvettes at 760 nm against blank using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. The experiment was performed in triplicate. The calibration curve was developedusing gallic acid in pure ethanol with the concentration in serial dilutions ranging from 4 to 1000 µg/ml as standard. The concentration of the samples were calculated according to the standard graph. Total phenol content was expressed as the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE/g).

Total flavones and flavonols

The total flavone and flavonol content was carried out as described by Miguel *et al.*[36] with slight modifications. Briefly, a volume of 0.4 ml of each extract was mixed with 0.4 ml of (2% AlCl3- 96% ethanol), and incubated for 1hr at room temperature. The absorbance was estimated in 1 ml cuvettes at 420 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. The experiment was performed in triplicates. The calibration curve was performed using quercetin in96% ethanol with serial dilutions ranging from 2 to 1000 µg/ml as standard. The concentration of the samples were calculated from comparison to thequercetin standard graph. Flavones and flavonol content was expressed as the milligrams of the quercetin equivalent per gram of the dry mass (mg QE/g).

Total antioxidant capacity [by ammonium molybdate reduction method]

Total antioxidant activity by molybdate assay was carried out as described by Pilar *et al.*[37] Briefly, molybdate reagent solution was prepared by diluting 500 ml of a solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate in distilled water in distilled water to make the total volume up to 1.5 L. A volume of 25 µl of each extract was added to 1 ml of molybdate reagent, then incubated at 95^{0}C for 90 min. The absorbance was estimated after cooling to room temperature in 1 ml cuvettes at 695 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. Mean values from three independent samples were calculated. The experiment was performed in triplicate. The calibration curve was performed using ascorbic acid in distilled water with the concentration in serial dilutions ranging from 0.04 to 10 mg/ml as standard. The concentration of the samples were calculated according to the standard graph. Total antioxidant capacity content was expressed as the milligrams of the ascorbic acid equivalent per gram of the dry mass (mg AAE/g).

DPPH scavenging assay

DPPH radical scavenging activity assay was carried out as described by Brand *et al.*[38] with slight modifications. Briefly, a volume 825 µl of 0.1 mM ethanol solution of 1,2-diphenyl-1-picrylhydrazyl (DPPH) solution) was added to 25 µl of a dilution series of the plant extracts ranging from (0.3-40 mg/ml) and mixed vigorously, then incubated in dark for 1hr. The absorbance was estimated in 1 ml cuvettes at 517 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer against ethanol blank where DPPH and extract were absent. The experiment was performed in triplicates and average absorption was noted for each concentrations. Butylated hydroxytoluene (BHT) was used as a standard. The percent inhibitions of the DPPH radical by the samples were calculated based on the below formula:

$$\frac{\text{(The absorbance of negative control} - \text{The absorbance of the sample)}}{\text{The absorbance of negative control}} \times 100$$

The standard graph was plotted against sample concentration taking the concentration of (BHT) as a positive control on the x-axis and the percentage scavenging activity on the y-axis. Based on this standard graph, IC_{50} was determined (concentration of sample able to scavenge 50% of DPPH free radical).

ABTS radical scavenging assay

The ABTS radical scavenging activity assay was carried out as described by Re *et al.*[39] with slight modifications. Briefly, a volume 7 mM of aqueous 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was added to an equal quantity of 2.4 mM K2S2O8 and incubated for 16 h in the dark to produce cationic ABTS. The resultant ABTS solution absorbance was adjusted to 0.7 units at 734 nm at room temperature. For each extract, 75 µl of a dilution series ranging from (1.25-40 mg/ml) were added to 825 µL ABTS solution and incubated for 5 min. The absorbance was estimated in 1 ml cuvettes at 517 nm against ethanol blank using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. The percentage inhibition calculated from the formula:

$$\frac{\text{(The absorbance of negative control} - \text{The absorbance of the sample)}}{\text{The absorbance of negative control}} \times 100$$

The standard graph was plotted against sample concentration taking the concentration of (BHT) as a positive control on the x-axis and percentage scavenging activity on the y-axis. Based on this standard graph, IC_{50} was determined (concentration of sample able to scavenge 50% of ABTS free radical).

Determination of reducing power

The reducing power activity assay was carried out as described by Oyaizu[40] with slight modifications. A volume of 50 µL of each extract was added to 200 µL of 0.2 M potassium buffer (pH 6.6) and 200 µL of potassium hexacyano ferrate (1% w/v), the mixture was vortexed and incubated for 20 min at 50^{o}C, followed by the addition of 200 µL of trichloroacetic acid (10% w/v), 600 µL of distilled water and 120 µL of ferric chloride (0.1%, w/v). The absorbance was estimated in 1 ml cuvettes at

700 nm against blank using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. The experiment was performed in triplicates and average absorption was noted for each concentrations. Ascorbic acid was used as a standard. The percentage inhibition calculated from the formula:

$$\frac{(\text{The absorbance of negative control} - \text{The absorbance of the sample})}{\text{The absorbance of negative control}} \times 100$$

The standard graph was plotted against sample concentration taking the concentration of ascorbic acid as a positive control on the x-axis and percentage scavenging activity on the y-axis. Based on this standard graph, IC_{50} was determined (concentration of sample able to scavenge 50% of Ascorbic acid free radical).

Statistical Analysis

Concentration-response curves were calculated using the Prism software package 5.00 for Windows, Graph Pad Software, San Diego, California, USA, http://www.graphpad.com/ (Graph Pad, San Diego, USA), and data were obtained from three independent experiments, each performed in triplicates (n =3) and represented as mean ± SD. Nonlinear best fit was plotted with mean ± SD. One-way ANOVA was performed followed by Tukey's multiple comparison tests. Throughout the analysis, $P< 0.05$ was considered significant.

RESULTS AND DISCUSSION

Asparagus aphyllus L.(AA),*Crataegus azarolus* L.(CA), and *Ephedra alata* Decne.(EA) extracts have been used in traditional Greco-Arab herbal medicine to treat various inflammatory diseases. However, few studies have been conducted to evaluate the effects of these extracts on inflammation. Macrophages are the major players of the innate immune response that promote inflammation via production of various key immune mediators, including cytokines such as TNF-α, IL-6 and chemokines, such as IL-8.[41,42] However, these same cells can adapt an anti-inflammatory behavior in order to stop the inflammatory process and retrieve a steady state through the secretion of anti-inflammatory cytokines, such as IL-10. The human THP-1 cell line and THP-1-derived macrophages have been reported to be useful cellular models for both host/pathogen interaction studies and anti-inflammatory drug screening.[43,44] Numerous scientific evidences indicate that the medical benefits of medicinal plants used by traditional healers, at least in part, are attributed to their significant antioxidant properties.[14] Therefore, in the first phase of the current study we measured the total phenolic contents of the test plants as well their antioxidant activity using three different test systems. In second phase, we used THP-1-derived macrophages as a monoculture and co-culture with hepatic cell line (HepG2) to assess the effects of the three plant extracts on the production of LPS-induced pro-inflammatory and anti-inflammatory cytokines.

Determination of total phenolic (TPC), total flavones and flavonols (TFFC), and total antioxidant capacity (TAC) content

Free radicals are implicated in distinct pathological conditions including but not limited to diabetes mellitus, cancer, and inflammation.[45] They damage key macromolecules especially lipids, proteins, and carbohydrates.[46] Fortunately, many vegetables, fruits and medicinal plants possess antioxidant phytochemicals, manly phenols and polyphenols (flavonoids, flavones and flavonols).[47] These compounds are also implicated in related biological effects, such as anti-inflammatory and anti-carcinogenic activities.[48]

The phenolic contents of the three extracts were estimated using the Folin-Ciocalteau method using gallic acid as a standard.This is arapid and simple method.However, it also measures nonphenolic compounds such as ascorbic acid, thiol, and nitrogen containing compounds.[38] Table 2 shows the phenolic contents in milligram gallic acid equivalents per gram (mg GAE/g) of dry plant extract. All three extracts contain relatively high phenolic contents. CA extract showed the highest content of 118 mg GAE/g extract. Similar TPC values were found in 50% ethanol extract of *Asparagus officinalis,*[49,50] *Cryptocarya alba* and *Crataegus monogyna.*[51]

Flavonoids are the main type of polyphenolic compounds that are known for their robust antioxidant activity.[35,52] The TFFC in the studied plants extract was determined based on quercetin as the reference compound and the TFFCexpressed in milligram quercetin equivalents per gram (mg QE/g) of dry extract. As for TPC, all three extracts contains relatively high TFFC and CA showed the highest value (4.8 mg QE/g) (Table 2). Similar results have been reported for *Asparagus officinalis.*[50,53]

The total antioxidant capacity (TAC) content of the plant extracts was estimated using ammonium molybdate reduction method withascorbic acid as standard. Table 2 presents the obtained TAC values in milligram ascorbic acid equivalents per gram (mg AAE/g) of dry plant. AA, EA, and CA extracts have relatively high antioxidant capacities. CA, as for TPC and TFFC, showed the highest TAC content (162 mg AAE/g extract).

In vitro antiradical activity

DPPH scavenging assay, ABTS radical scavenging assay, and reducing power (RP) were used here to assess the free radical scavenging capacity of the three plant extracts. DPPH, a stable dark purple free radical, turns into a yellow stable diamagnetic molecule upon reaction with antioxidants.[38] The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm induced by anti-oxidants. DPPH exerts a rapid hydrogen accepting ability. As such it reacts with antioxidants (converting into 1,1-diphenyl-2-picrylhydrazine), resulting in a decrease in absorbance. The degree of discoloration indicates the scavenging potential of the anti-oxidant extract. AA, EA and CA extracts exhibited significant radical scavenging activity. The maximum activity was found in CA extract as the IC_{50} value was about 56µg/ml (Table 3). This may be due to relatively high levels of phenols, flavones and flavonol (Table 2), which are known for their antioxidant activity. The IC_{50} values for AA and EA extracts were 440 and 100 µg/ml respectively (Table 3).

ABTS is a blue radical, which is converted into a colorless form in the presence of a hydrogen donor.[39] The ABTS IC_{50} value was the lowest for CA 131 µg/ml. The ABTS IC_{50} values for AA and EA extracts were 669 and 154 µg/ml, respectively (Table 3).

In the reducing power(RP) assay, the iron (Fe^{+3}) in ferric chloride is oxidized to ferrous (Fe^{+2}) ion by antioxidant molecules, converting the solution color from yellow to green which expresses the efficiency of RP of the plant extract contents.[56] The RP IC_{50} of AA, EA and CA were 630, 530 and 50 µg/ml, respectively (Table 3). CA extracts contain relatively high levels of phenols and significant antioxidant activity as measured with RP, DPPH, and ABTS.

Cytotoxic effects

To make sure that the plant extracts concentrations applied in the following experiments are safe and non-toxic, the MTT and LDH (LDH-release test) assays were used to assess cell viability post treatment withAA-extract,CA-extract, and EA-extract. MTT and LDH assay were carried out 24 h after treatment with increasing concentrations (1-250 mg/mL of culture medium) of the three plant extracts. The MTT assay is designed to be used for the quantification of both cell proliferation and cell viability in cell population using 96-well plate format. This test is widely used in the *in vitro* evaluation of cytotoxic and cytostatic effects of plant extracts. Membrane integrity can be evaluated by measuring the lactate dehydrogenase activity. Lactate dehydrogenase, an enzyme located in the cytoplasm, catalyses the conversion of lactate and pyruvate.

Table1: Ethno-botanical data, collection time and parts used of the three medicinal plants used in the present study

Plant species	Voucher specimen code	Family	English Name	Arabic Name	Time of collection	Used parts	Medicinal use
Asparagus aphyllus L.	(Pharm-PCT-249)	Asparagaceae	Prickly Asparagus	Halyoon	April–July (2015)	Aerial parts	Liver diseases, diarrhea and dysentery
Ephedra alata Decne.	(Pharm-PCT-904)	Ephedraceae	Camel's ephedra	O'leak or Alanda	April–July (2015)	Aerial parts	Anti-cancer, asthma and respiratory problems
Crataegus azarolus L.	(Pharm-PCT-712)	Rosaceae	Azarole	Zaa'roor	April–July(2015)	Leaves	Cardiovascular diseases, sexual weakness, cancer and diabetes

Table 2: Phenol, flavones and flavonol content, and phosphomolybdateassay results (total antioxidant capacity) (mg/g of plant)

Sample	Phenols	Flavones and flavonol	TAC
AA	12.09 ± 0.12^{c}	3.81 ± 0.06^{b}	43.1 ± 0.66^{c}
EA	30.53 ± 0.67^{b}	1.76 ± 0.09^{c}	72.07 ± 2.99^{b}
CA	117.97 ± 2.19^{a}	4.79 ± 0.07^{a}	162.58 ± 5.38^{a}

Values in the same column followed by the same letter are not significantly different by the Turkey's multiple range test ($p<0.05$); data are the means of three replicates. ND, not detected.

Table 3: Antioxidant activities (DPPH, ABTS and Reducing power) expressed as IC_{50} (µg/ml).

Sample	DPPH	ABTS	Reducing power
AA	440 ± 12^{b}	669 ± 6^{a}	630 ± 7^{a}
EA	100 ± 4^{c}	154 ± 1^{b}	530 ± 1^{b}
CA	56 ± 0.1^{a}	131 ± 2^{c}	50 ± 1^{c}
BHT	9 ± 0,1^{d}	3 ± 0,1^{d}	ND
Ascorbic acid	ND	ND	3 ± 0,1^{d}

Values in the same column followed by the same letter are not significantly different by the Turkey's multiple range test ($p<0.05$); data are the means of three replicates. ND, not detected.

When lactate dehydrogenase is found within the media on the cells, there are two possible causes: The first is cellular death and the second may be a 'leak' in a cell membrane. When cells are disrupted, the lactate dehydrogenase activity (in the media) is elevated. Cell viability was higher than 90% in all extracts concentrations tested (up to 125 mg/ml) in the mono- and co-culture systems (Figure 1), except for CA (~85%) and EA (~80%) in THP-1 mono culture (Figure 1A). Based on the MTT and LDH results, we decided to exclude concentrations of 250mg/ml and to use concentrations up to 125 mg/ml in the following experiments.

Effects on the secretion of pro-inflammatory cytokines IL-6 and TNF-α

Pro-inflammatory cytokines, TNF-α, and IL-6 are prominent mediators of inflammatory diseases.[42,57,58] In recent years, various medicinal plant-derived products have been reported to modulate the mRNA and protein secretion of pro-inflammatory cytokines as well as anti-inflammatory cytokines. For example, flavonids, such as a moradicin, genistein, and silybin reduce TNF-α production from LPS-treated RAW 264.7 cells.[41] Baicalininhibitsthe induction of IL-1, IL-6, TNF-α, and IFN-G at protein as well as at RNA levels from human staphylococcalentero toxin-stimulated monocytes.[42] Here, we show that EA, CA and AA -extracts could modulate the production of pro-inflammatory cytokines (TNF-α and IL-6) as well as anti-inflammatory cytokine (IL-10) in the LPS-activated THP-1-derived macrophages in monoculture as well as in a co-culture system. THP-1-derived macrophages produce detectable amounts of IL-6 and TNF-α after stimulation with LPS. Maximal TNF-α, IL-6 and IL-10 concentrations were detectable in the culture supernatants 4 h, 6 h, 24 h post LPS stimulation, respectively (data not shown). Therefore, these time points were used to assess the effect of the three plant extracts on TNF-α, IL-6, and IL-10 production in cultures of THP-1 (Figure 2 and Figure 3). Treatment of macrophages with the three plant extract alone did not affect the levels of any of the studied cytokines (Data not shown). TNF-α secretion into the culture supernatant of untreated and LPS treated THP-1 cells and their co-cultures with HepG2 are depicted in Figures 2A, 3A, 4A. All three extracts inhibited the secretion of the TNF-α. The inhibition effects were more obvious in co-culture than in mono-cultures. The secretion of IL-6 was also affected by the plant extracts. CA-extract (Figure 2B) and AA-extract (Figure 3B) inhibited significantly the secretion of IL-6 in both culture systems. No significant effects were detected after treatment with EA-extracts (Figure 4B).

These results suggest that the plant extracts probably exert anti-inflammatory effects through the inhibition of IL-6 and TNF-α production. Similar results were found using different medicinal plants. For example, *Hypericumtriquetrifolium* inhibited the production of NO, TNF-α, and the expression of iNOS, but not of IL-6.[42] Feverfew extracts were found to effectively reduce LPS-mediated TNF-α and CCL2 (MCP-1) releases by THP-1 cells.[42,59] *Peganumharmala*-extract was found to exert anti-inflammatory properties throughout increasing the release and expression of IL-10 mRNA as well as by suppressing IL-1, IL-6 and TNF-α mRNA expression in THP-1 cells.[44]

Effects on the secretion of anti-inflammatory cytokine IL-10

IL-10 is a potent anti-inflammatory cytokine. It limits and terminates inflammatory responsesthrough the inhibition of the production of pro-inflammatory cytokines.[60] Injection of the recombinant IL-10 causesa decrease in the blood level of TNF-α which has proven beneficial for such diseases.[61,62] Indeed, several inflammatory diseases share the dual characteristic of a very low level of IL-10 and a high blood level of TNF-α. The results obtained here indicate that LPS treatment of THP-1-derived macrophages cells induced the secretion of a low level of cytokine IL-10. Treatment of THP-1-derived macrophages and their co-cultures with HepG2 with AA-extract (Figure 3C) significantly increased IL-10 protein secretion, EA-extract (Figure 4C) significantly increased IL-10

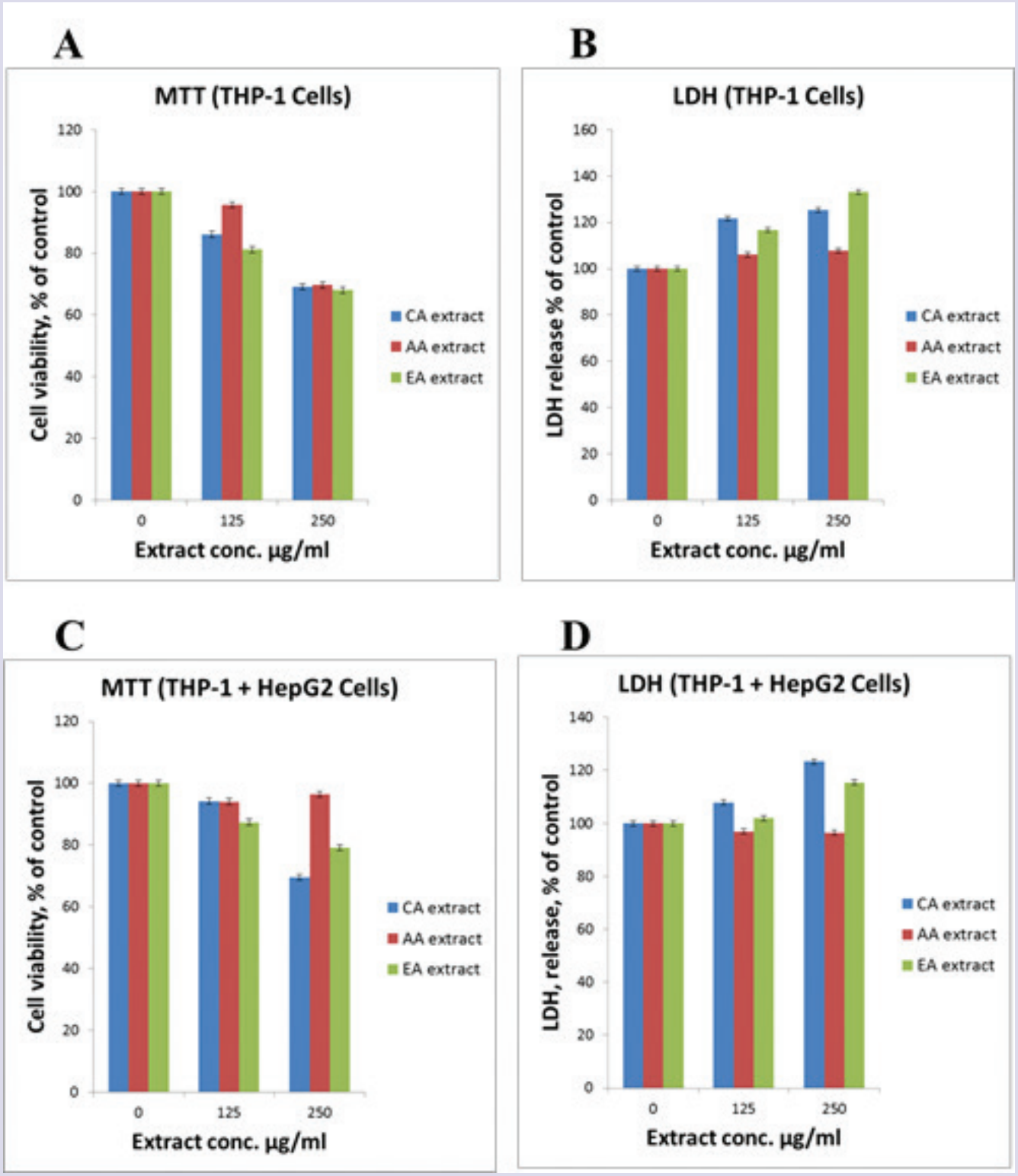

Figure 1: MTT and LDH cytotoxicity assays in, (A and B) THP-1-derived macrophages and, (C and D) their co-cultures with HepG2 cells. After 24hours of treatment with increased concentrations of extract (0-250 µg/mL) from *Crataegus azarolus* L.(CA),*Asparagus aphyllus* L.(AA), and*Ephedra alata* Decne.(EA). The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. LDH activity was determined at 490 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of plant extracts treated cells relative to untreated cells. Values represent means ± SD ($P < 0.05$ was considered significant compared to control) of three independent experiments carried out in triplicates.

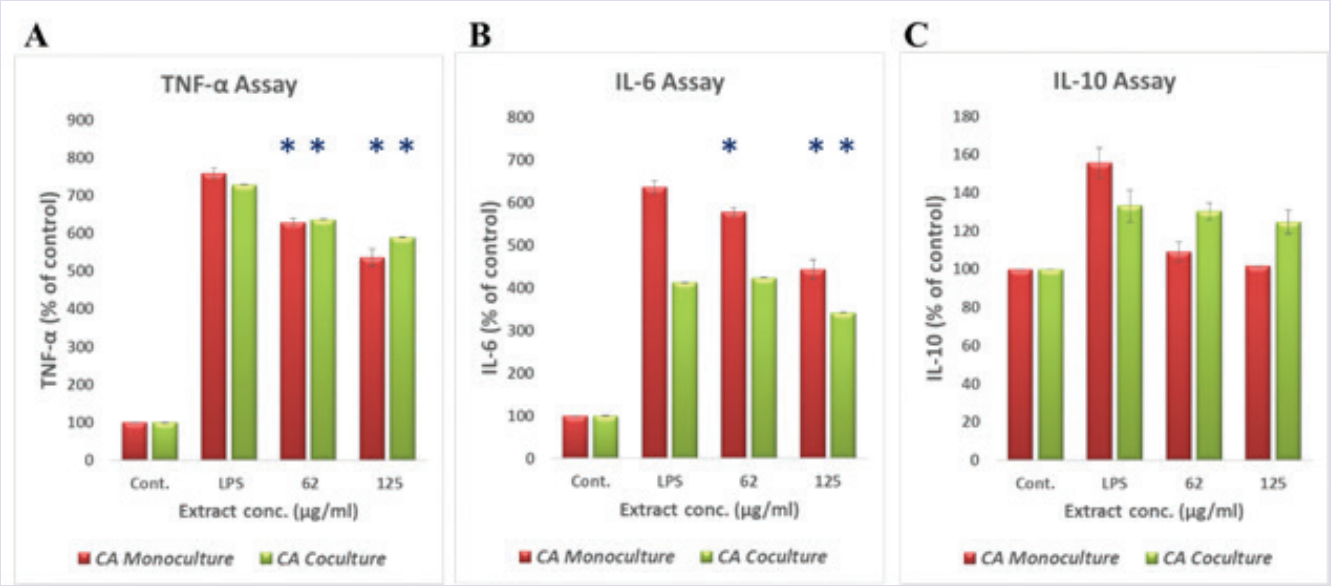

Figure 2: Effects of *Crataegus azarolus* L.(CA),water/ethanol extract on the anti-inflammatory mediators (A):TNF-*α*,(B): IL-6 and pro-inflammatory mediator (C): IL-10 release by LPS-activated THP-1 cells (monoculture system) or THP-1 and HepG2 cells (coculture system). Cells were pretreated with a non-toxic concentration, 0 (negative control) or different concentrations (62.5, and 125 *µ*g/mL of plant extract), the cells then treated with 1 *µ*g/mL LPS. Positive control wascarried out by LPS with no plant extract. The level of TNF- a, IL-6 and IL-10 release was measured by an ELISA kit after 4 h, 6 h or 24 h of treatment respectively. Experiments were performed in triplicate. Bars indicate the percentage of means and standard deviations. Values represent means ± SD (*$P< 0.05$ was considered significant compared to control) of three independent experiments carried out in triplicates.

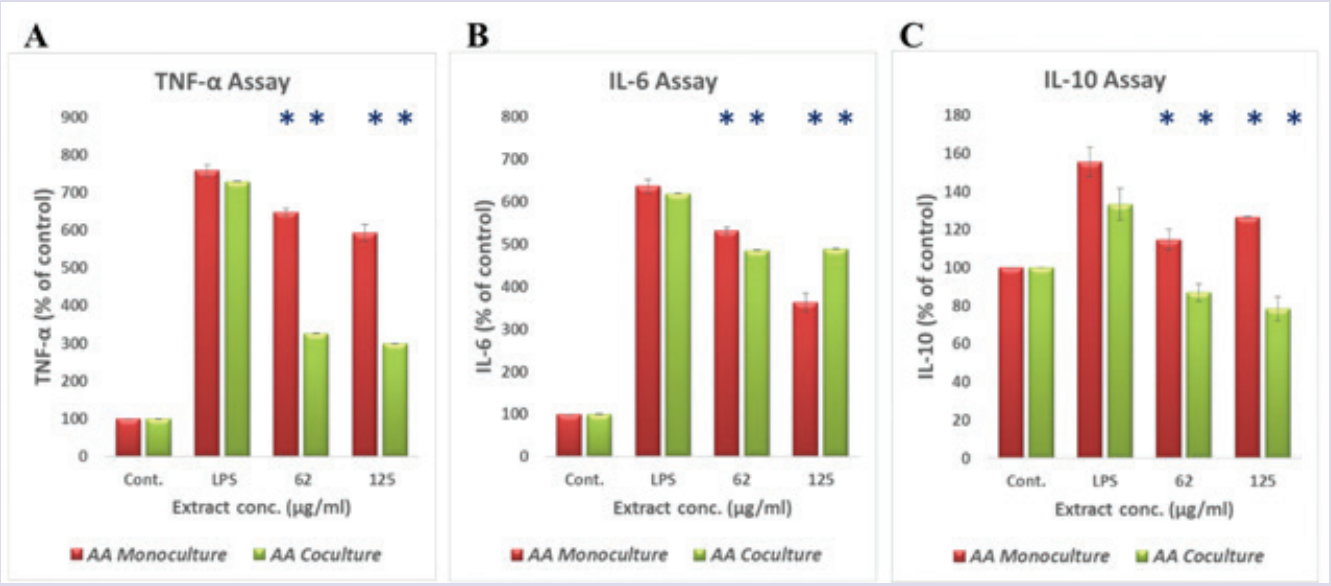

Figure 3: Effects of *Asparagus aphyllus* L.(AA),water/ethanol extract on the anti-inflammatory mediators (A):TNF-*α*,(B): IL-6 and pro-inflammatory mediator (C): IL-10 release by LPS-activated THP-1 cells (monoculture system) or THP-1 and HepG2 cells (coculture system). Cells were pretreated with a non-toxic concentration, 0 (negative control) or different concentrations (62.5, and 125 *µ*g/mL of plant extract), the cells then treated with 1 *µ*g/mL LPS. Positive control wascarried out by LPS with no plant extract. The level of TNF- a, IL-6 and IL-10 release was measured by an ELISA kit after 4 h, 6 h or 24 h of treatment respectively. Experiments were performed in triplicate. Bars indicate the percentage of means and standard deviations. Values represent means ± SD (*$P< 0.05$ was considered significant compared to control) of three independent experiments carried out in triplicates.

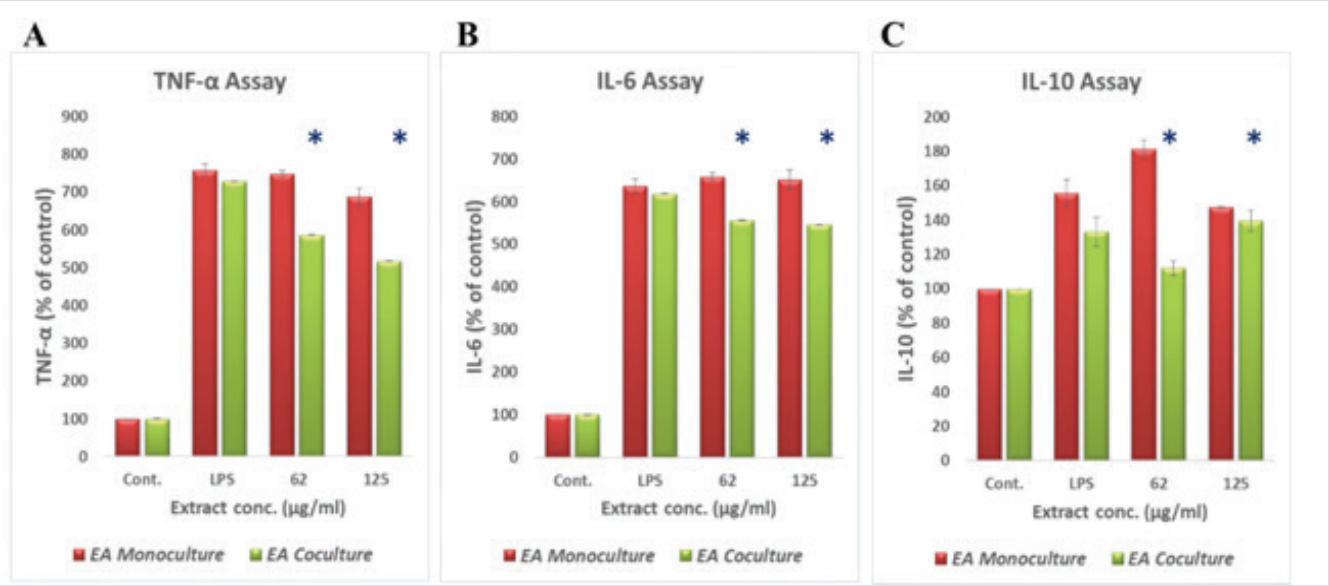

Figure 4: Effects of *Ephedra alata* Decne. (EA),water/ethanol extract on the anti-inflammatory mediators (A):TNF-*α*,(B): IL-6 and pro-inflammatory mediator (C): IL-10 release by LPS-activated THP-1 cells (monoculture system) or THP-1 and HepG2 cells (coculture system). Cells were pretreated with a non-toxic concentration, 0 (negative control) or different concentrations (62.5, and 125 *µ*g/mL of plant extract), the cells then treated with 1 *µ*g/mL LPS. Positive control wascarried out by LPS with no plant extract. The level of TNF- a, IL-6 and IL-10 release was measured by an ELISA kit after 4 h, 6 h or 24 h of treatment respectively. Experiments were performed in triplicate. Bars indicate the percentage of means and standard deviations. Values represent means ± SD (*P<0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

protein secretion in co-culture system but no notable elevation in mono-culture system. No significant effects were seen after treatment with CA-extracts (Figure 2C). These effects were evident at the same extract concentrations as the maximal inhibitory concentrations of IL-6 and TNF-α. Previous reports have clearly shown the antagonist effect of IL-10 on the secretion of pro-inflammatory cytokines,[61,62] suggesting that AA-extract and EA-extract mediated inhibition of the LPS-induced secretion of IL-6 and TNF-α may pass through the induction of IL-10 secretion. Similar results were observed after treatment of THP-1 cells with *Peganum harmala.*[44] Furthermore, IL-10 was found to inhibit the production of pro-inflammatory cytokines[61,62] and injection of exogenous recombinant form of IL-10 was shown to decrease the blood level of TNF-α.[63,64]

The ability of AA-extract and EA-extract to modulate both, the pro-inflammatory and anti-inflammatory cytokines by LPS-activated THP-1-derived macrophages represents an additional argument for the suggestion that it is an alternative or a complement that may help in the treatment and/or prevention of inflammatory diseases.

CONCLUSION

Asparagus aphyllus L.**,** *Crataegus azarolus* L., and *Ephedra alata* Decne. have been used in traditional Greco-Arab herbal medicine to treat inflammatory diseases. However, to our knowledge, no scientific evidences regarding their anti-inflammatory effects have been previously published. Results obtained here suggestthat the traditional uses of three plant extracts as anti-inflammatory remedy may be mediated, at least, by their significant antioxidant capacity and inhibition of pro-inflammatory as well as anti-inflammatory cytokine secretion.

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CONFLICT OF INTEREST

Nil

ABBREVIATION USED

Nil

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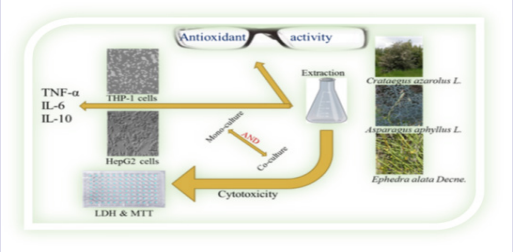
PICTORIAL ABSTRACT



SUMMARY

Summary: Results obtained here using mono-and co-cultures of hepatocytes and monocytes suggest that the traditional uses of three plant extracts as anti-inflammatory remedies may be mediated by their significant antioxidant capacity, inhibition of pro-inflammatory cytokines secretion concomitant with enhancement of anti-inflammatory cytokines secretion.

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