**Introduction**

A very large number of individuals around the world still rely on traditional medicine for the treatment of various acute and chronic diseases. Even in countries where the modern medicine is very advanced, these herbal extracts, syrups, essential oils and other formulations are sold in pharmacies and supermarkets. Botanical supplements are believed to be safe as there are no or minimal side-effects. Moreover, using herbal medicines is advantageous due to the occurrence of several biologically active compounds which in certain cases may act synergistically to deliver an effective curing power that cannot be attained by any one component in the natural mixture. On the other hand, they could also be harmful because of the possible presence of toxic components (Ekor 2014).

The genus *Arum*, belonging to the Araceae family, has a long history of traditional medicinal uses in the Middle East, southern Europe and northern Africa. In folk medicine, *Arum* species such as *A. dioscorides* (Afifi-Yazar et al. 2011) and *A. palaestinum* (Afifi-Yazar et al. 2011; Zaid et al. 2012; Naseef et al. 2017) have been used for the treatment of cancer. Cuckoo pint (*A. maculatum*) is a woodland plant that grows in the northern region of Iraq where it is commonly known as ‘*kardi*’. Cuckoo pint leaves are the edible parts consumed by the local population after removing the toxic constituents by cooking. Toxicity of *A. maculatum* is attributable to the presence of toxic volatiles, especially amines, oxalates, cyano compounds as well as contents such as alkaloids and saponins (Azab 2017). The raw plant is not edible due to its toxic and allergic properties. It has been reported to cause irritation to skin, mouth, tongue, and throat, resulting in throat swelling, difficulty in breathing, burning pain, and stomach ache (Robertson 2009). However, its therapeutic importance for many diseases, including kidney
and liver injuries, hemorrhoids and as a pain reliever has been reported (Abbasi et al. 2014; Kochmarov et al. 2015). Mice orally treated with A. maculatum methanolic extract showed different histological and antioxidant activity effects in the liver (Kadri et al. 2016). Other studies have shown antimicrobial and antifungal activities of this plant extract against a wide range of Gram-positive, Gram-negative bacteria and fungi (Safari et al. 2014; Colak et al. 2009). The pro-inflammatory activity of monocot lectin isolated from A. maculatum has been reported to increase neutrophil migration (Alencar et al. 2005). The antitumor activity of A. palaestinum on different human cancer cells has been investigated by Farid et al. (2015), where significant anti-proliferative activity was reported. TNF-α initiate inflammation, either local or systemic, via stimulation of cytokines such as interleukin-1β (IL-1β) and IL-6. Other roles are the signal suppression of T-cell and the inhibition of antigen production by dendritic cells (O’Shea et al. 2002). IL-1β induces both local and systemic signs of inflammation. It also promotes the production of various enzymes, for example, cyclooxygenase type 2, adhesion molecules and importantly other cytokines and chemokines of pathogenic importance in some diseases (Dinarello et al. 2009). The present study aimed to investigate the active compounds in the aqueous extracts of A. maculatum leaves as well as their antioxidant, anti-inflammatory and cytotoxic effects.

**Materials and Methods**

**Chemicals and reagents**

Cell culture reagents were purchased from Lonza (Slough, Berkshire, UK). Culture plates were obtained from Becton Dickinson, Roswell Park Memorial Institute (RPMI 1640 medium was purchased from Gibco (Paisley, UK). All other reagents and chemicals [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Fetal bovine serum (FBS) trypsin, DPPH and MTT] were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse IL-1β and TNF-α were obtained from Pierce Berkshire, UK). Culture plates were obtained from Bec-

**Plant collection and extract preparation**

*A. maculatum* leaves were obtained from a local market in the Kurdistan region located north of Iraq and was identified by an eminent plant taxonomist at Baghdad University. Voucher specimens were deposited at the Herbarium of Bakrajo Agricultural Technical Institute (Sulaimani Polytechnic University, Kurdistan, Iraq). Leaves were air dried at room temperature, and then grounded into powder. The aqueous extraction method was selected to prepare A. maculatum extract because it is a commonly used method in folk medicine. The extract was prepared as follows (Taskeen et al. 2009): 50 g of powdered dried leaf powder was suspended in 500 ml of distilled water in conical flasks and stirred for 6 h using a magnetic stirrer. The extracts were filtered using filter paper (Whatman No. 1) and centrifuged at 3000 rpm for 10 min. The supernatants were then transferred and dried under reduced pressure using a rotary evaporator and were kept at 4 °C for further analysis and experiments.

**GC-MS analysis and identification of phytochemical components**

The GC-MS analysis of aqueous cuckoo pint (*A. maculatum*; ‘kardi’) extract was performed using a Clarus 500/580 Perkin Elmer GC (Connecticut, USA), including an AOC-20i auto-sampler, and equipped with a fused silica capillary column Elite-1 (100% methyl polysiloxane; 30 m × 0.25 mm, 0.25 µm). Helium (99.99%) was used as a carrier gas at a constant flow rate of 1 ml/min. 0.5 µl samples were injected at a split ratio (1:10). Injector temperature was 280 °C. Oven temperature was programmed to automatically increase at a rate of 10 °C/min from 110 °C up to 200 °C, then at 5 °C/min further up to 280 °C (10 min). Mass scans were taken at electron energy 70 eV (0.5 sec scan interval) and in the range of 40–450 Da; with a total run time of 36 min. Analysis of mass spectrum of GC-MS was done using the database of the National Institute Standard and Technology (NIST). The mass spectrum of the unknown components was compared with the spectrum of the components stored in the NIST library (Nezhadali et al. 2010; Sathyaprabha et al. 2011).

**Antioxidant activity measurement**

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Mimica-Dukic et al. (2003). The DPPH assay has been widely used to detect the scavenging activities of plant extracts. The principle of the assay is based on reduction of DPPH via hydrogen donation from antioxidant by which the color change can be spectrophotometrically recorded. Briefly, 1 ml of the samples was mixed with an equal volume of the DPPH solution (60 µM). After 30 min incubation at 37 °C in darkness, the absorbance was recorded at 517 nm spectrophotometrically (Perkin-Elmer Lambda 25, Germany). L-ascorbic acid was used as a positive control and measurements were carried out in triplicate. Inhibition of free radicals by DPPH was calculated by the following equation:

\[
\text{DPPH scavenging activity (\%)} = \frac{\text{Ac-As}}{\text{Ac}} \times 100
\]

Where, Ac = control absorbance and As = sample absorbance.
**Cell culture**

The murine cell line L20B was provided by the Center of Biotechnology at Al-Nahrain University (Baghdad, Iraq). L20B cell line is derived from mouse cells (fibroblasts) and has been found to express the human poliovirus receptor (Pipkin et al. 1993). RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% antibiotic (containing 10 000 U/ml penicillin G, 10 mg/ml streptomycin and 25 μg/ml amphotericin B) was used for cell culture and maintenance. Cells were incubated at 37 °C in humidified 5% CO₂ and cultured to 2.5 × 10⁵ cells/ml concentration.

**Measurement of cell viability**

The colorimetric cell viability MTT assay was carried out as described before (Nouri et al. 2015). Cells were seeded in 96-well plates at a concentration of 1 × 10⁴ cell/ml. After 48 h of incubation, 100 µl of plant extracts at concentrations of 0.4, 4, 40 and 400 µg/ml were added to each well followed by an incubation period of 24 h. After the incubation, 10 µl of MTT solution (5 mg/ml) was added to each well and the plates were further incubated at 37 °C for 4 h. Finally, 50 µl of dimethyl sulfoxide (DMSO) was added to each well and incubated for 10 min. L20B cells cultured in medium without *A. maculatum* extract served as the control. The absorbance was measured at 620 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Percent of inhibition ratio was calculated according to the following formula:

\[
G1\% = \left( \frac{\text{OD of control wells} - \text{OD of test wells}}{\text{OD of control wells}} \right) \times 100
\]

Where GI = growth index and OD = optical density.

**Detection of pro-inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA)**

Protein levels of TNF-α and IL-1β in the serum were measured using an ELISA kit (Pierce Endogen, Rockford, IL, USA) according to the manufacturer instructions. Briefly, 50 µl/well of samples (treated mice with 50 and 100 µg/ml and control mice which received normal saline solution of 0.9% NaCl) was added to the anti-mouse TNF-α and IL-1β-pre-coated 96-well plates. After washing with phosphate buffered saline (PBS, containing 0.05% Triton X-100), the detection antibodies (0.5 µg/ml) were dissolved in 2% bovine serum albumin (BSA) in PBS and then added to the plates before incubation for 4 h at room temperature. After washing, 50 µl of conjugate streptavidin-horseradish peroxidase (HRP) dissolved in 2% BSA (in PBS) (1:250) was added to each well. After washing with the buffer, 100 µl of chromogenic substrate tetramethylbenzidine (TMB) was added to each well before incubation for 15 min at room temperature to allow color development. To stop the reaction, the HRP was denatured by adding 1 M sulfuric acid (50 µl/well) resulting in a color change to yellow. Absorbance was read at 450 nm using a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMax Pro software version 5.4. The cytokine concentrations were determined by comparison with the values for standard recombinant human cytokines (0-5 ng/ml) and all samples were analyzed in triplicate.

**Statistical analysis**

Statistical analysis was performed using SPSS software version 16.0. All results are presented as means ± standard error. Significance was calculated using analysis of variance (ANOVA) and Fisher Least Significant Difference (LSD) test.

**Results and Discussion**

**GC-MS analysis**

GC-MS analysis of the leaf extracts showed the presence of major components (peak area > 1%). The major compounds identified are: 9-octadecenoic acid, methyl ester, (E) (24.76%), hexadecanoic acid, methyl ester (22.45%), followed by benzene propanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester (17.74%). These constituents have been investigated for their biological and therapeutic properties, including the anti-inflammatory (Othman et al. 2015), antioxidant (Pinto et al. 2017), antibacterial and antifungal activities (Agoramoorthy et al. 2007). Previous investigation has shown that palmitic acid induces apoptosis in the human leukemic cell line and demonstrated in vivo antitumor activity in mice (Harada et al. 2002). Our results are supported by those of a previous report (Kianinia and Farjam 2018) of the essential oil of Iranian *A. maculatum* extracts, mainly consisting of palmitic acid, phytol, methyl 9,12,15-octadecatrienoate and methyl linolenate.

**Antioxidant activity**

The aqueous extract was tested for its antioxidant activity at concentrations of 100, 150, 200, and 250 µg/ml using DPPH. Results showed that the extracts showed a significantly higher and concentration-dependent radical scavenging activity up to 200 µg/ml, as compared with the positive control (L-ascorbic acid). Higher activity (P ≤ 0.01) was observed at 250 µg/ml while the lowest activity was observed at 100 µg/ml (P ≤ 0.05) compared with the ascorbic acid control (Fig. 1).

The antioxidant activity of phenols and flavonoids has been attributed to their redox activities, allowing them to act as reducing agents and free radical scaveng-
The observed DPPH scavenging activities indicate an antioxidant protective role against oxidative stress, which is implicated in several diseases. The DPPH assay has been widely used to detect the radical scavenging activity of extracts of several medicinal plant species, including *Panax ginseng* (Ali et al. 2006), *Echinacea purpurea* (Paek et al. 2009), *Withania somnifera* (Dewir et al. 2010) and *Albizia odoratissima* (Banothu et al. 2017). The results reported herein suggest that *A. maculatum* leaf extracts contain hydrogen donating compounds that can eliminate free radicals and therefore could explain their therapeutic application for different pathological injuries caused by oxidation.

**Cell viability**

Our results showed that treatment with *A. maculatum* extract significantly (*P* ≤ 0.01) inhibited cell growth as compared to control cultures (Fig. 2). The highest inhibition (69% ± 0.024) was recorded at 400 µg/ml and it decreased when extract concentration was reduced, reaching only 45% ± 0.020 cell growth inhibition at 0.4 µg/ml. Therefore, the suppression of LB20 cell proliferation was concentration dependent. These results might be due to the presence of flavonoids, glycosides, polyphenols and saponins which act as active agents against cell death. It has been previously reported that fatty acids may play a role in triggering apoptosis associated with trauma in the central nervous system (CNS) and peripheral nervous system (PNS) (Ulloth et al. 2003). Our results indicated that the major compounds present in *A. maculatum* extract are fatty acids, including hexadecanoic acid, palmitic acid (a saturated fatty acid), and 9-octadecenoic acid, methyl ester, (E) (elaidic acid), an unsaturated fatty acid.

Generally, fatty acids and their methyl ester derivatives are biologically active compounds with antioxidant, anticancer and antihistaminic properties (Melariri et al. 2012). Palmitic acid has been reported earlier as a potential anticancer drug (Harada et al. 2002) as significant loss of viability of nerve growth factor (NGF)-differentiated PC12 cells was observed after 24 h treatment with stearic and...
Determination of pro-inflammatory cytokines
ELISA showed that the mean concentrations of the two cytokines (IL-1β and TNF-α) were dose-dependent (Fig. 3). Mice treated with A. maculatum extract at 100 ng/ml showed significantly (P ≤ 0.05) decreased TNF-α cytokine levels, whereas 50 ng/ml caused no significant reduction. However, treatment with 50 and 100 ng/ml increased IL-1β level in the blood and significantly higher cytokine levels (P ≤ 0.05) were observed with 100 ng/ml, as compared to controls.

A. maculatum agglutinin reportedly shows both dose-dependent and independent pro-inflammatory activity, inducing neutrophil migration by the resident cells and, in the presence of macrophages, respectively (Alencar et al. 2005). It has been reported that the underlying mechanism of the anti-inflammatory and anti-microbial activities of flavones are due to their ability to regulate the Toll receptor/ NFκB axis which is responsible for the expression of mediator inflammation such as TNF-α, IL-1β and cyclooxygenase-2 (Jiang et al. 2016). Gupta et al. (2010) proposed that the small molecules that suppress NF-κB activation might have a prospective therapeutic potential by phosphorylation of the NFκB p65 subunit. Inhibition of p65 phosphorylation reduces the expression of inflammatory cytokines, limiting characteristic cell damage of acute inflammation.

Conclusion
Determination of pro-inflammatory cytokines, MTT assay and DPPH tests revealed that A. maculatum leaf extracts possess anti-inflammatory, cytotoxic and anti-oxidant activities. Therefore, our findings substantiate A. maculatum as a potentially useful herb in alternative medicine against some serious human health problems. Further research on cytotoxicology is required to guarantee herbal drug safety.

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References
Inhibiting NF-κB activation by small molecules as a therapeutic strategy. Biochim Biophys Acta 1799:775-787.