

Full Length Research Paper

Antimicrobial and cytotoxic activities of methanol extract of *Alhagi maurorum*

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***Alhagi maurorum* Medik (Fabaceae) is the single species of genus *Alhagi* found in Iraq, locally known as Aqual. Various studies have shown *A. maurorum* used in folk medicines. However, its potential health benefits have not been studied in details. Methanol extracts of *A. maurorum* from the aerial part were screened for total phenolic and flavonoids contents, antioxidant, antimicrobial and cytotoxic activities. The total phenolic and total flavonoids contents were assessed by Folin–Ciocalteu and aluminum nitrate methods, respectively. Antioxidant properties were measured by diphenyl-1-picrylhydrazyl (DPPH), linoleic acid peroxidation and xanthine oxidase. Antimicrobial activity against six microorganisms was tested using disc diffusion method and cytotoxicity test was carried out using methyl thiazolyl tetrazolium (MTT) on the human leukemia cell line (HL-60). The antioxidant properties and total phenolic contents of the leaves were higher than those of the flowers. Antimicrobial activities were characterized by inhibition zones and minimum inhibition concentration (MIC) ranged between 58.0-80.7 and 60.4-84.0 $\mu\text{g mL}^{-1}$, respectively. Leaves and flowers extract induced inhibitory effect against the proliferation of HL-60 cells and IC_{50} was 16.0 and 22.0 $\mu\text{g mL}^{-1}$, respectively. The antimicrobial and cytotoxicity of extracts seemed to be positively correlated with their antioxidant potentials. The present findings indicated that leaves and flowers extracts are highly cytotoxic to HL-60 cells, and leaves extract was more potent in this regard. The present study suggests that *A. maurorum* may be used as possible natural antioxidant, antimicrobial, and an effective therapeutic agent in the management of acute promyelocytic leukemia.**

Key words: *Alhagi maurorum*, phenolic content, antioxidant, antimicrobial, cytotoxic.

INTRODUCTION

Natural products perform various functions, and many of them have interesting and useful biological activities (Harvey, 1999). There are more than 35,000 plant species being used in various human cultures around the world for medicinal purpose. Researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs against cancer, as well as viral and microbial infections (Hoffmann *et al.*, 1993; Harvey, 1999; Srinivasan *et al.*, 2001). According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for

their primary healthcare needs. Use of herbal medicine in Asia represents a long history of human interactions with the environment. Plants used in traditional medicine contain a wide range of ingredients that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants accumulated in areas where the use of plants is still of great importance (Diallo *et al.*, 1999).

The medicinal value of plants lays on some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive



Figure 1. Aerial parts of *A. maurorum*.

compounds of plants are the alkaloids, tannins and phenolic compounds (Edeoga et al., 2005). Furthermore, previous as well as recent investigators reported that phenols, polyphenol, and flavonoids are natural antioxidant plant products that have been found in various concentrations in most medicinal plants as well as other plant kingdom (Rice-Evans et al., 1995; Middleton et al., 2000; Scalbert et al., 2005; Kiselova et al., 2006; Borchardt et al., 2008; Parthasarathy et al., 2009; Tarawneh et al., 2010). They also indicated that these compounds have been shown to possess various therapeutic values such as antibacterial, anti-inflammatory, antiaging, antimutagenicity and anticarcinogenic activities by reducing the oxidative damage or stress induced by free radical species.

Several species of family Fabaceae have been explored revealing the presence of phenolic compounds (Al-Yahya et al., 1987). *Alhagi maurorum* Medik is the single species of genus *Alhagi* (Fabaceae) found in Iraq, locally known as Aqual. *A. maurorum* is a spiny deep-rooted, rhizomatous, perennial shrub, with roots that can extend six or seven feet into the ground. Leaves deciduous simple, small, present at base of each side twig, obovate to oblong, shortly petiolate, with rounded tip, up to 2 cm. Flowers solitary or in pairs in axils and along twigs, with deep red to purple papilionate petals. The spiny, intricately-branched shrub reaches 1.5 to four feet in height (Figure 1).

The plant is mentioned in the Qur'an as a source of sweet Manna (Kahrizi et al., 2012). It has also been used as a sweetener. Various studies have shown that *A. maurorum* has been used in folk medicines. It is used in diaphoretic, diuretic, expectorant and anti-ulcer treatments (Uphoff, 1959; Chakravarty, 1976; Zakaria et al., 1999). The plant is also used as laxative and in the treatment of

diseases of the urinary tract and liver (Marashdah et al., 2008). Oil from the leaves of the plant is used for the treatment of rheumatism while the flowers of the plant are used for the treatment of piles migraine, and warts (Atta et al., 2010; Kahrizi et al., 2012). However, to date the potential health benefits of *A. maurorum*, have not been studied in detail. In this study, *A. maurorum* was collected to determine antioxidant, antimicrobial and cytotoxic activities and to determine the phenolics level responsible for these properties.

MATERIALS AND METHODS

Chemical reagents

All chemicals and reagents were of analytical grade and obtained from either Sigma-Aldrich or Merck Chemical Co. (Darmstadt, Germany).

Collection and extraction of *Alhagi maurorum* Medik

The areal parts (leaves and flowers) were collected during the period 2010-2011 from Al-Tarmyiah town which is located in region 60 km north-east Baghdad, Iraq. The plant material was identified by Dr. Ali Al-Mosawy, department of Biology, College of Science, University of Baghdad, Baghdad, Iraq. The fresh aerial parts were washed thoroughly with tap water at room temperature to remove dirt prior to the drying process. These washed aerial parts were dried in the shade at room temperature for seven days. Then, they were crushed into fine powder. The powder was placed once in the Soxhlet cold extractor using 80% methanol as solvent and was kept here for three consecutive days. The extracts were concentrated to dryness in rotary evaporator under reduced pressure at 45°C. The extraction and evaporation procedures were repeated three times. Then, the resulting extract was stored, protected from light in a refrigerator at 4°C in a glass container until use.

Determination of total phenolics spectrophotometrically

Total phenolic compound concentrations were determined spectrophotometrically (Mohammadzadeh et al., 2007). Briefly, 1 mL of extract was mixed with 1 mL of Folin Ciocalteu reagent. After 3 min, 1 mL of saturated sodium carbonate solution (20%) was added to the mixture and adjusted to 10 mL with distilled H₂O. The reaction mixture was kept in the dark for 1 h with intermittent shaking. The absorbance was measured at 725 nm using a spectrophotometer. Phenolic contents were calculated on the basis of the standard curve of Gallic acid and three measurements were performed to obtain a mean value.

Determination of total flavonoid content spectrophotometrically

Plant extracts were separately re-dissolved in 95% ethanol at a concentration of 50 mg mL⁻¹. Methanol extracts (0.1 mL) were diluted with 80% aqueous ethanol (0.9 mL). Aliquots of diluted extracts (0.5 mL) were added to test tubes and mixed with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate and 4.3 mL of 80% ethanol. After standing for 40 min at room temperature, the absorbance of the reaction mixtures was measured at 415 nm (Nieva Moreno et al., 2000). Quercetin was used as a standard compound to construct a standard curve and three measurements were performed to obtain a mean value.

Free radical scavenging activity

The free radical scavenging activities of extracts were measured with DPPH (Kumar et al., 2008). The DPPH radical has a deep violet color due to its unpaired electron and radical scavenging capability can be followed spectrophotometrically by absorbance loss at 517 nm through producing the pale yellow non-radical. Based on this assay, equal volumes (0.5 mL) of DPPH (60 µM) and plant extracts (10, 50 or 100 µg mL⁻¹) were mixed in a cuvette and allowed to stand for 30 min at room temperature. Then, the absorbance was read at 517 nm in a UV/VIS Lambda 19 spectrophotometer.

Induced lipoperoxidation

The antioxidant capacity of plant extracts of different concentrations (10, 50 or 100 µg mL⁻¹) to inhibition of linoleic acid peroxidation were assayed using the ferric thiocyanate method (Yen et al., 2003). Briefly, an aliquot of the sample in distilled water was mixed with 5 volumes of 0.02 M linoleic acid emulsion and 4 volumes of 0.2 M phosphate buffer (pH 7.0) in a test tube. The mixture was then placed in the dark at 4°C for 8 days to accelerate lipid oxidation. After addition of the ferric chloride and thiocyanate solutions, the peroxidation value was measured by the absorbance at 500 nm and three measurements were performed to obtain a mean value.

Xanthine oxidase activity

Xanthine oxidase activity was evaluated spectrophotometrically by following the formation of uric acid at 292 nm (Russo et al., 2000). The assay mixture contained, in a final volume of 1 mL, 50 mM phosphate buffer pH 7.8, 25 mM solution of xanthine and 24 mU xanthine oxidase (specific activity 1 U mg⁻¹ of protein). Different concentrations of plant extracts (10, 50 or 100 µg mL⁻¹) were added to samples before the enzyme and their effect on the generation of uric acid was used to calculate regression lines. The results were expressed as percentage of inhibition enzyme activity.

Antimicrobial activity test

Staphylococcus aureus (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 15523), *Salmonella typhimurium* (ATCC 13311) and *Candida albicans* (ATCC 10231) were used for antimicrobial activity test. Antimicrobial activity of *A. maurorum* extract was investigated by the disc diffusion method according to the method of Bauer et al. (1966). The antimicrobial screening was performed using Mueller-Hinton agar (MHA, Oxoid) and MHA with 0.5 mL defibrinated sheep blood for bacteria, and Sabouraud Dextrose agar (SDA, Oxoid) for a yeast. The methanol extracts were dissolved in 10% DMSO, then discs (6.0 mm diameter) submerged with 100, 200 and 400 µg mL⁻¹ were placed on the inoculated plates. Similarly, for each plate, a blank disc was carried by adding solvent alone (10% DMSO) to act as negative controls. Seeded agar plates were inoculated with 0.1 mL of inoculum. Discs were then placed on the seeded agar plates, and the plates were incubated at 37°C for 24 h for bacteria, and 48 h for *C. albicans*. After the incubation, the zones of growth inhibition around the discs were measured. All of the experiments were conducted in triplicate. The results are reported as the average of three experiments.

Minimum inhibitory concentration (MIC) determination

The MIC of leaves and flowers extracts against each of the tested isolates were determined by the macrobroth dilution method. Serial dilutions and concentrations at ranges 10- 90 µg mL⁻¹ were prepared in nutrient broth in the case of bacteria and liquid yeast extract medium in the case of *C. albicans*. The tubes were then inoculated with 0.2 µL of cultures (10⁶ cells). Uninoculated tubes containing growth medium or growth medium and extract were used as controls. The tubes were then incubated at appropriate temperatures for 24 h. The MIC value was determined as the lowest concentration of the methanol extract in the broth medium that inhibited the visible growth of the test microorganism (NCCLS, 2000).

Growth inhibition activity for HL-60

In this study, cancer cell growth inhibition activity was measured by using MTT assay (Banskota et al., 2000). To 180 µL aliquots of human leukemia HL-60 cells were seeded in a 96 well polystyrene tissue culture plates at the concentration of 5 x 10⁵ cells mL⁻¹ using RPMI 1640 containing 2 mmol L⁻¹ L-glutamine, supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37°C. After 16 h, the plant extract solutions were added to the wells at the concentrations of 10, 20 and 40 µg mL⁻¹. The cells were then further incubated for an additional 24, 48 and 72 h at 37°C in the same conditions. After incubation, 20 µL of the labeling mixture, consisting of 5 mg mL⁻¹ MTT in phosphate-buffered saline solution (PBS) were added to each well and incubation was continued for 4 h at 37°C and then culture medium was removed and 200 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 min to dissolve air bubbles. The culture plate was placed on a Biotex Model micro-plate reader and the absorbance was measured at 550 nm. The amount of color produced is directly proportional to the number of viable cell. The IC₅₀ was performed using concentrations ranges 1-320 µg mL⁻¹. All assays were performed in three replicates for each concentration.

Statistical analysis

The results are reported as mean ± SD of three independent repli-

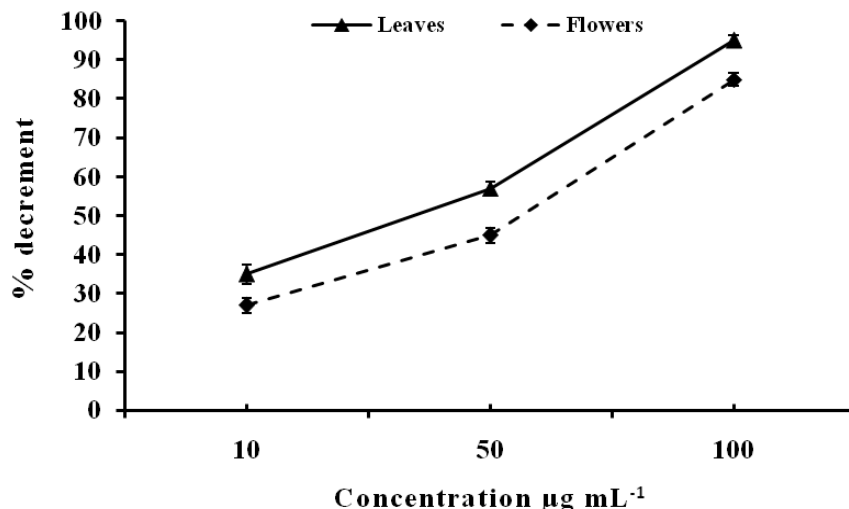


Figure 2. DPPH free radical scavenging activity of *A. maurorum* extracts. Each value represents the mean \pm S.D. of three experiments.

Table 1. Total phenolic and flavonoid contents (mg g⁻¹ crude extract) determined by Folin–Ciocalteu and aluminium nitrate colorimetric methods of *A. maurorum* extracts.

<i>A. maurorum</i> Extract	Total Phenolics Content (mg g ⁻¹) *	Total Flavonoids Content (mg g ⁻¹) **
Leaves	50.39 \pm 2.67	39.24 \pm 1.54
Flowers	32.00 \pm 1.62	18.50 \pm 0.80

* Results are presented as mean \pm SD (n = 3) and calculated as gallic acid equivalents.

** Results are presented as mean \pm SD (n = 3) and calculated as quercetin equivalents.

catates. Statistical analysis of data was carried out by computer using SPSS version 11.5 software. Level of significant was assessed by using the Analysis of Variance (ANOVA) test. The level of significance was shown using the least significant difference (LSD) test.

RESULTS

Total phenolics and flavonoids were identified in leaves and flowers extract of *A. maurorum* using spectrophotometric methods, and they are given in Table 1. The total content was extract-dependent. The highest total content for phenolics and flavonoids were observed in leaves extract (50.39 \pm 2.67; 39.24 \pm 1.54, respectively) and then followed by flowers extract (32.00 \pm 1.62; 18.50 \pm 0.80, respectively).

The free radical scavenging effect of *A. maurorum* extracts in DPPH was determined, and given as a percentage of free radical scavenging activity for three concentrations 10.0, 50.0 and 100.0 μ g mL⁻¹ of each extract (Figure 2). In general, a concentration-dependent was observed, and 100 μ g mL⁻¹ was significantly better than the other two concentrations for the two investigated extracts. In this regard, leaves extract had strong free radical scavenging activity of about 95.00%. As shown in Figure 2, the flowers extract also showed antioxidant

activity, but that was less than observed in leaves extract with 82.00 % of free radical scavenging activity.

Figure 3 shows the effect of different concentrations of *A. maurorum* extracts on linoleic acid peroxidation activity. From the figure, it is clear that leaves extract showed a more potent capacity to suppress lipid peroxidation when compared with flowers extract and concentration-dependent effects were observed. Also, 100 μ g mL⁻¹ was significantly better than the other two concentrations for the two investigated extracts. The inhibition activities were (80.00 and 70.00%, respectively).

The effect of both *A. maurorum* extracts with their concentrations on xanthine oxidase activity are shown in Figure 4. The addition of the leaves extract tested determined a dose-dependent inhibition of xanthine oxidase activity, but the addition of flowers extract exhibited a lower effect. Higher concentrations showed more efficient inhibitory action on xanthine oxidase activity than other lower concentration. The inhibition activities were (92.00 and 80.00%, respectively).

The agar-disc diffusion method was used to determine the inhibition zones of the leaves and flowers ethanolic extracts from *A. maurorum*. The two Gram-positive, two Gram-negative bacterial strains and a yeast were used.

According to the results in Figures 5 and 6, two extracts

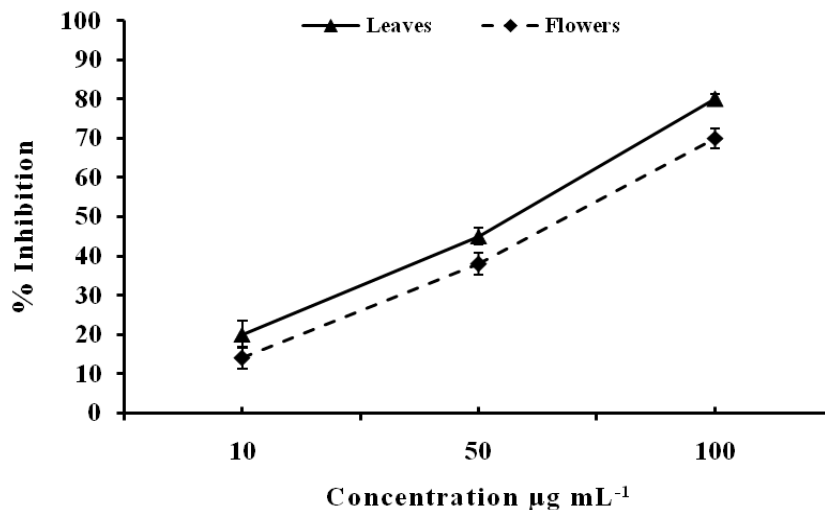


Figure 3. Effect of *A. maurorum* extracts on linoleic acid peroxidation. Each value represents the mean \pm S.D. of three experiments.

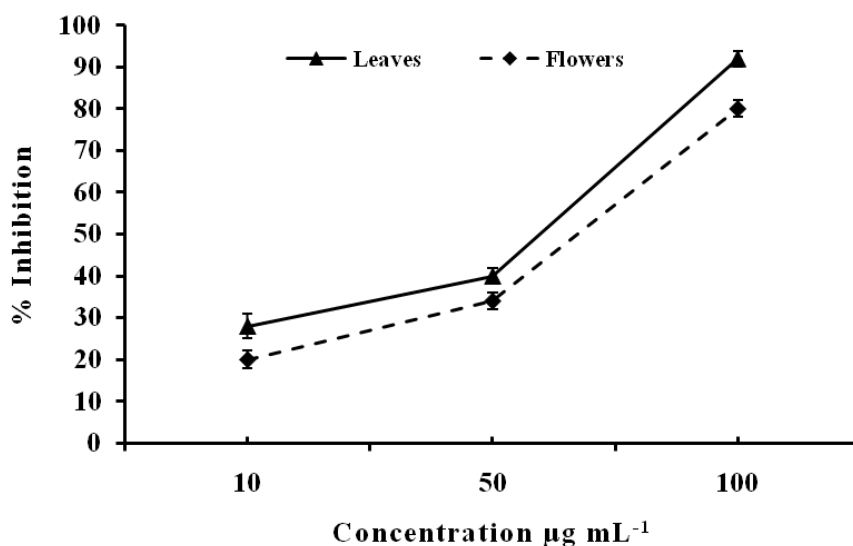


Figure 4. Effect of *A. maurorum* extracts on xanthin oxidase activity. Each value represents the mean \pm S.D. of three experiments.

showed antibacterial activity against *S. aureus*, *B. subtilis*, *P. auerginosa*, *S. typhimurium* and *C. albicans*.

As shown in Table 2, the MIC of leaves extract was 58.0, 58.8, 60.6, 68.8 and 80.7 $\mu\text{g mL}^{-1}$, respectively. Whereas, the antibacterial activity in flowers extract was 60.4, 62.4, 65.2, 65.0 and 84.0 $\mu\text{g mL}^{-1}$, respectively.

The cytotoxic activities of *A. maurorum* extracts on the HL-60 cell line was further studied to evaluate the dose related cytotoxic activities. The inhibitory effect was determined at 24, 48 and 72 h later and presented in Figures 7 and 8. Leaves extract (10, 20 and 40 $\mu\text{g mL}^{-1}$) showed significant cytotoxicity on HL-60 cell line and the dose 40 $\mu\text{g mL}^{-1}$ in three recommended times was more

potent in this regard, which showed 45.76, 23.98 and 17.42%, respectively as compared with the controls (Figure 7). With respect to flowers extract the same effect was observed, but had less antiproliferative activity when compared with the leaves extract, which showed 60.76, 43.21 and 20.57%, respectively as compared with the controls (Figure 8). The IC_{50} of leaves extract on HL-60 was 16 $\mu\text{g mL}^{-1}$ and 20 $\mu\text{g mL}^{-1}$ for flowers extract (Table 3).

DISCUSSION

Plants are important source of potentially useful structures

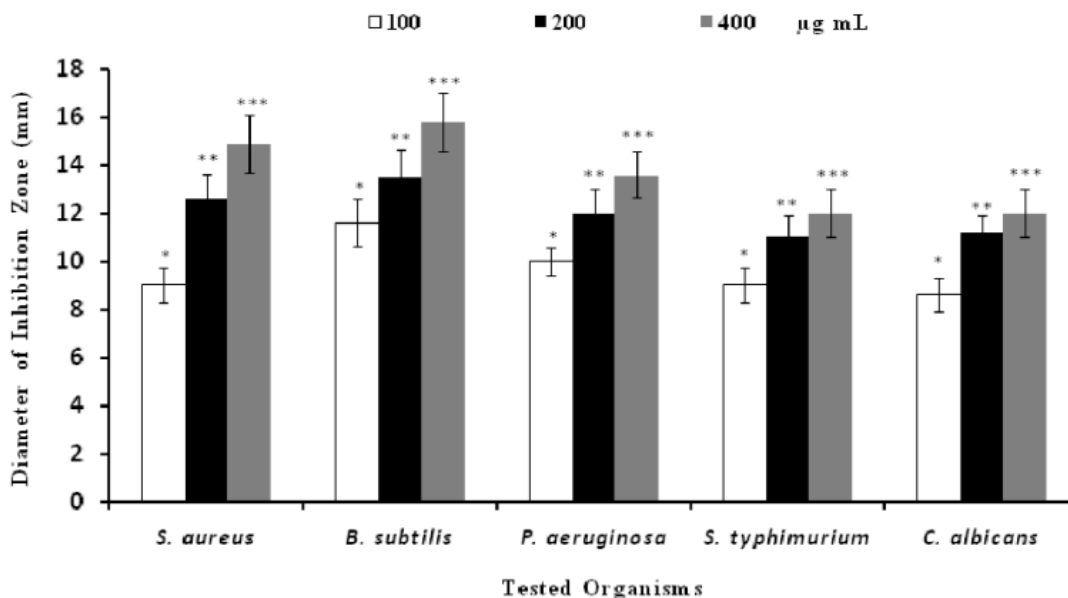


Figure 5. Antimicrobial activity (zone of inhibition) of *A. maurorum* leaves extract at different concentrations on five tested organisms. Data is expressed as the mean ± S.D. of data obtained from triplicate experiment and significantly different by one way ANOVA followed least significant difference (LSD). *P < 0.05, **P < 0.01, ***P < 0.001, compared to the control group.

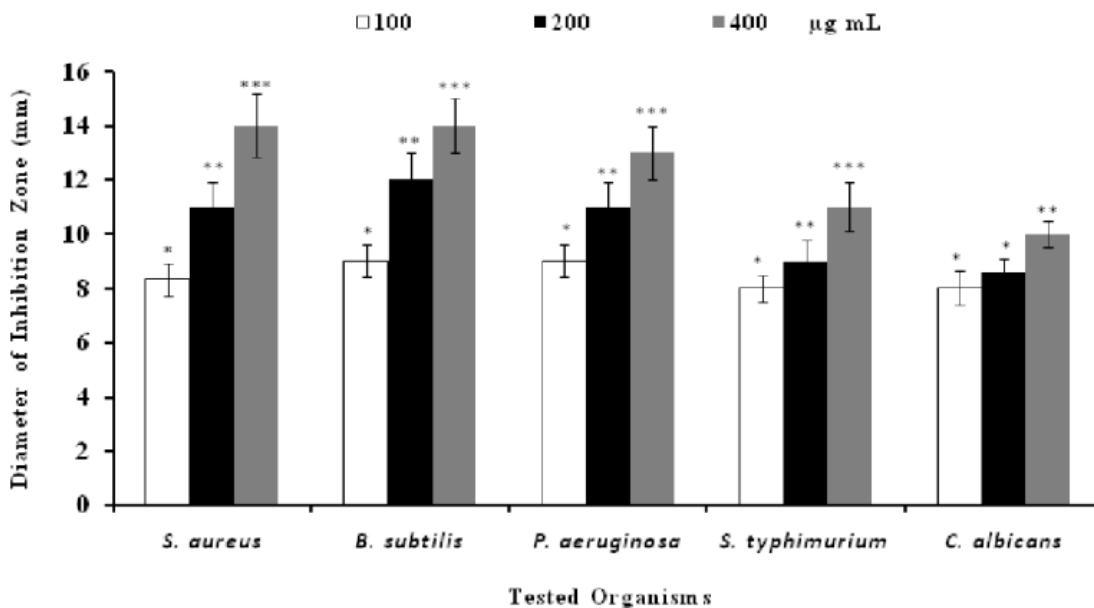


Figure 6. Antimicrobial activity (zone of inhibition) of *A. maurorum* flowers extract at different concentrations on five tested organisms. Data is expressed as the mean ± S.D. of data obtained from triplicate experiment and significantly different by one way ANOVA followed least significant difference (LSD). *P < 0.05, **P < 0.01, ***P < 0.001, compared to the control group.

Table 2. Minimum Inhibitory Concentration (MIC µg mL⁻¹) of *A. maurorum* extracts.

<i>A. maurorum</i> Extract	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>S. typhimurium</i>	<i>C. albicans</i>
Leaves	58.0±6.3	58.8±6.1	60.6±8.3	68.8±4.6	80.7±4.5
Flowers	60.4±5.6	62.4±5.0	65.2±6.2	65.0±2.7	84.0±0.0

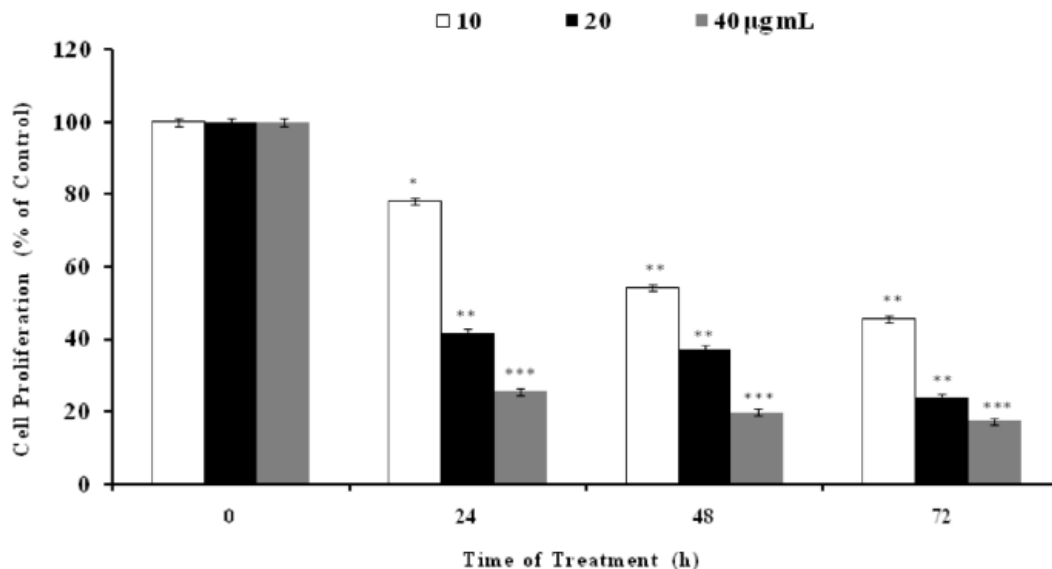


Figure 7. Antiproliferative activity of *A. maurorum* leaves extract at different concentrations on HL-60 cells. Data is expressed as the mean \pm S.D. of data obtained from triplicate experiment and significantly different by one way ANOVA followed least significant difference (LSD). *P < 0.05, **P < 0.01, ***P < 0.001, compared to the control group.

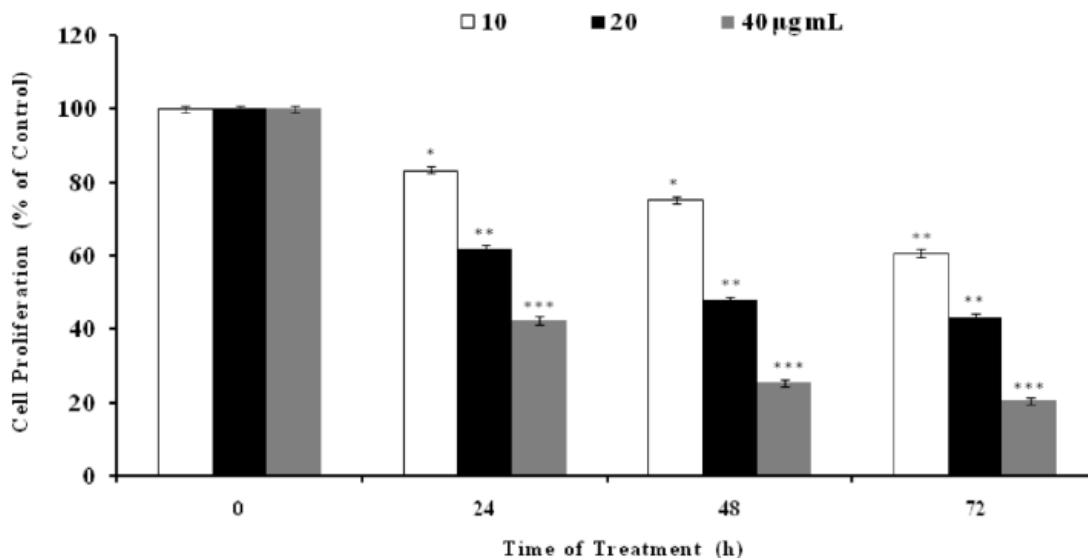


Figure 8. Antiproliferative activity of *A. maurorum* flowers extract at different concentrations on HL-60 cells. Data is expressed as the mean \pm S.D. of data obtained from triplicate experiment and significantly different by one way ANOVA followed least significant difference (LSD). *P < 0.05, **P < 0.01, ***P < 0.001, compared to the control group.

for the development of new chemotherapeutic. The current study addresses the total phenolics antioxidants, antimicrobial and cytotoxic effects of locally plant *A. maurorum* selected from Al-Tarmiyah which is located north-east Baghdad of Iraq. The selected plant was based on its traditional uses as folk medicinal plant for the treatments of various types of diseases in many countries including Iraq (Marashdah et al., 2007). The question is;

how does *A. maurorum* exert its effects? These effects can be justified on the ground of chemical compounds, which makes the medicinal importance of the plant. Phytochemical screening of *A. maurorum* extract revealed the presence of flavonoids, glycosides, alkaloids, saponins, tannins, steroids, and anthraquinone as major constituents (Abdel Rahman et al., 2011; Samejo et al., 2012). The additional constituents were reported in *A.*

Table 3. Inhibitory Concentrations (IC_{50} $\mu\text{g mL}^{-1}$) of *A. maurorum* extracts.

<i>A. maurorum</i> Extract	IC_{50}
Leaves	16.0 \pm 2.4
Flowers	22.0 \pm 3.5

maurorum extract: β -sitosterol, cinnamic acid, coumaric acid, and hydroxybenzoic acid (Ahmad et al., 2009). Therefore, the chemistry of plant natural products and their biological effects have been the potential of intensive research, and the effects are explained in terms of chemical constituents.

The spectrophotometric investigation of the methanol extract found that the leaves and flowers extract had higher phenolic and flavonoid contents and exhibited strong antioxidant activity that assessed using three different methods. The anti-oxidant properties and total phenolic contents of the leaves were higher than those of the flowers. Furthermore, a positive correlation has been shown between anti-oxidant activities assays and total phenolics, indicating that these compounds are more likely to contribute to the antioxidant potential of the investigated plant extracts (Choi et al., 2002; Miliauskas et al., 2004; Sakanaka et al., 2005; Parthasarathy et al., 2009). Thus, the phenolic compounds might contribute directly to anti-oxidative action of plant extract (Kumar et al., 2011; Laghari et al., 2012), and the anti-oxidant activity of *A. maurorum* extracts may play an important role in their anti microbial and anti-proliferative activities.

The antimicrobial activity has also shown to increase as the concentration of this plant extracts increases. The obtained result is in accordance with that achieved by Abd-Ellatif et al. (2011) on *Aspergillus flavus*, *A. alternata*, *F. oxysporum*, *F. solani*, *Bipolaris oryzae*, *Chetomium* sp. and *Mucor* sp. In another study, the ethanolic extract of the *A. maurorum* showed significant antimicrobial activity against Gram negative, Gram positive bacteria as well as unicellular and filamentous fungi (Zain et al., 2012). Furthermore, it has some medicinal properties such as anti-oxidant, anti inflammatory (Awaad et al., 2011), anti ulcerogenic (Awaad et al., 2006) and anti-diarrhoeal activity (Gutierrez et al., 2007).

The activity of *A. maurorum* plants could be explained, at least by their antimicrobial properties, due to their high flavonoid contents. The possible mechanism of the antimicrobial action may be related to viability inhibition of the tested organisms by *A. maurorum* extract due to the loss of their ability to bind to DNA. This fact suggested that *A. maurorum* extract might act by inhibiting DNA replication and cell reproduction. These phenolics interfere not only with the propagation but also with the formation of free radicals both by chelating transition metals and by inhibiting enzymes involved in the initiation reaction (Russo et al., 2002). It has been reported that oxidative stress induced by reactive oxygen species may cause different

sub-cellular damages such as lipid peroxidation. Consequently, these highly reactive free radicals have been implicated in pathology of different number of diseases in humans such as neurodegenerative disorders, *diabetes mellitus*, atherosclerosis, Parkinson disease and cancer (Tarawneh et al., 2010).

The *in vitro* cytotoxicity assessment of a human acute myeloid leukemia cell line (HL-60) revealed that the effects were dose and time-dependent. For the best knowledge of the investigator, this task has not been targeted. However, the cytotoxicity of *A. maurorum* extracts seemed to be positively correlated with polyphenolic compounds, and the results of antioxidant activity evaluation support such correlation. In agreement with such theme, it has been reported that plant phenol compounds are well known that these products are powerful anti-oxidants. It also reported that antioxidant compounds such as polyphenols, flavonoids and others play a vital role in removing free radicals and in cytotoxic effects (Sulaiman et al., 2011).

The cytotoxic effects of phenolic compounds may depend on their lipophilicity, which is very important for the penetration into cells. On the other hand, lipids and proteins present in biological membranes facilitate the solubility of polyphenols, and differences in cell membrane structures and metabolic activation of chemicals can also affect the activity of polyphenols (Szliszka et al., 2009). Different mechanisms linked to phenolics mediated cytotoxicity may be combined with structure activity potentials, including proteasome inhibition (Chen et al., 2005), inhibition of fatty acid synthesis (Brusselmans et al., 2005), topoisomerase inhibition (Yoon et al., 2007), induction of cell cycle arrest (Haddad et al., 2006), accumulation of p53 (Singh and Agarwal, 2006) or enhanced expression of c-fos and c-myc (Ganguly et al., 2005).

However, the precise mechanism of HL-60 death remains to be determined. Further studies are underway to explore the molecular mechanisms of the *A. maurorum* extracts, and to determine its properties *in vivo*. The present investigation support the use of methanol extract of *A. maurorum* as a strong disinfectant in folk and modern medicine, and the antioxidant activity which may justify its use as a source of natural oxidant to protect humans from deleterious oxidative processes including cancer.

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