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EVALUATION OF ALUMINUM STRESS INDUCED ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES IN ROMAN NETTLE

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ABSTRACT

In this current work, Roman nettle (*Urtica pilulifera* L.), a traditional medicinal plant that is very common and widespread species throughout Asia, Europe, and Northern Africa, was used as a model plant to investigate changes in antimicrobial activity following the application of aluminum stress. *U. pilulifera* seedlings were grown in growth-room conditions and 0, 100, and 200 µM $AlCl_3$ were applied to the plants together with Hoagland solution (20 ml) for two months. The antimicrobial activities were tested against nine strains of bacteria (*Salmonella* sp., *Staphyllococcus aureus*, *Escherichia coli*, *E. coli* O157:H7 and *Bacillus cereus*) and fungus (*Penicillum* sp., *Saccharomyces cerevisiae*, *Candida tropicans* and *C. albicans*) by using the disc diffusion and agar well methods. The accumulated Al was measured by using ICP-OES in the leaves of studied plant samples. Additionally, a control group (water + 11.31 mg l^{-1} Al) was prepared and applied to selected bacteria and fungi in order to understand the reason for obtained antimicrobial activities of Roman nettle is whether because of the compounds isolated from nettle leaves exposed to Al stress, or Al itself accumulated in leaves. The data proved that inhibitory antimicrobial effects were altered in *U. pilulifera* upon the application of Al stress, especially on fungi species.

KEYWORDS:
Urtica pilulifera, aluminum stress, antimicrobial activity

INTRODUCTION

The most common source of antimicrobial compounds are plants. Popularity of their utilization as health remedies having minimal side effects grows globally [1]. The species of *Urticaceae* family are very common and show widespread distribution around the borders of arable fields, gardens, roads and forests naturally throughout Europe, Asia and Northern Africa. Chemical compositions of the family members are very rich and have been used as herbal medicines [2]. *Urtica pilulifera, U. dioica*, and *U. urens* are the only *Urticaceae* family member species found in Anatolia. Modes of actions and chemical properties of these species are very similar and they are used as substitutes of each other. The leaves of *Urtica* have biological compounds, including histamine, formic acid, acetylcholine, acetic acid, butyric acid, leukotrienes, 5-hydroxytryptamine, and other irritants [3]. They are also rich in minerals, chlorophyll, amino acids, lecithin, carotenoids, flavonoids, sterols, tannins and vitamins. The roots of *Urtica* contain chemical constituents such as scopoletin, sterols, fatty acids, polysaccarides and isolecithin [4].

Urtica sp. is used for antibacterial and antifungal studies. Fatty acids obtained from *Urtica* were screened for their antibacterial and antifungal activities and found to be effective against ATCC strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphyllococcus aureus*, *Bacillus subtilis*, and *Enterecoccus faecalis* as well as *Candida albicans* and *C. parapsilosis* [5]. Another study indicated that *U. dioica* having antibacterial properties was active against *Salmonella enteritidis*, *S. gallinarum*, *S. agalactiae*, *K. pneumoniae*, *S. aureus*, and *E. coli* [6]. In a similar study, moderate antimicrobial effects of the water extract of *U. dioica* against the ATCC strains and their clinical isolates of ten bacteria and one fungus (*C. albicans*) were shown [7]. A similar moderate antimicrobial activity was observed when crude extracts of *U. crenulata* were used against *S. typhi*, *Shigella flexneri*, and *S. sonnei* [8]. Also, UDA (*U. dioica* agglutinin) from *U. dioica* possesses both antifungal and insecticidal activities [9]. Furthermore, antiviral compounds from *U. diocia* are heavily used in the medical sector. For example, mannose specific plant lectins serve as potent and selective inhibitors for human immunodeficiency virus [2].

Al, considered as one of the inhibiting factors is toxic for plant growth and development [10]. It decreases the overall leaf number, size and shoot

biomass, inhibits the root elongation and causes the chlorosis and necrosis in leaves, leading to the reduced photosynthetic activity [11]. In addition, it causes cellular and ultrastructural modifications in leaves as a result of inhibition of cell division and elongation [12-13].

Plants are the targets of continuous attempts of attack by microbes and against invaders plants deploy the diverse strategies [14]. One of the strategies plants exert is synthesizing substances such as phenolics and polyphenols, flavonoids, terpenoids, alkaloids and essential oils, lectins, tannins, polypeptides, serving as the components of plant defense mechanisms [15-16]. In general, those substances show antimicrobial and antifungal activities against intruding agents [17]. As mentioned above, Al may cause alterations in pathways and coordinated behaviors of cells and may lead to changes in plants by affecting the flow of metabolites through the pathways. *U. pilulifera* (Roman nettle) is one of the important traditional medicinal plants in Turkey and the source of the antibacterial and antifungal compounds [4]. In this study, *U. pilulifera* was used as study material for the investigation of the possible effects of different levels of Al exposures on antibacterial and antifungal activities of *U. pilulifera*.

MATERIALS AND METHODS

Surface sterilization of *U. pilulifera* seeds was done by immersing in %50 ethyl alcohol for 1 min. and then rinsed in de-ionized water for 5 min. For germination, seeds were transferred into small pots containing sterilized compost. Seeds were periodically moisturized with de-ionized water during two weeks' germination period. As the shoots of young plantlets were 3-4 cm long, they were transferred into standard pots containing 210 g of sterilized compost. Then, plantlets were grown in normal growth room conditions. Each of the experimental groups of eight replicates was watered with Hoagland full strength nutrient solution [18] (40 ml in each) containing 0 (control), 100 and 200 µM $AlCl_3$ at two-day intervals for the 2 months. The soil pH was adjusted to 5 for Al treatments using 0.2 % (v/v) H_2SO_4. The plants were grown under fluorescent tubes giving an irradiance of 5000 lx (day/night - 16/8 respectively), temperature of 23 ± 2°C and relative humidity 45-50 % [19].

Seedlings were harvested at the end of the two-month experiment period. After harvesting, the fresh leaves of *U. pilulifera* were cleaned with de-ionized water and dried in the shade at room temperature. Thereafter, the dried leaves (5 g were used for each experimental group) were ground into powdered form using a cutting mill and then dried powdered leaf materials were extracted with chloroform at 60°C in a Soxhlet apparatus. The chloroform was removed from the leaf extracts by means of vacuum distillation at 35°C using a Rotary Evaporator (BUCHI Rotary Evaporator Rotavapor R-210/R-215 model). The remaining aqueous solutions were referred to as the crude extracts. All extracts were stored in a refrigerator for further antimicrobial activity study.

All test organisms were obtained from the Gebze Institute of Technology, Department of Molecular Biology and Genetics, Gebze-Turkey and were as follows: *Salmonella* sp., *Staphyllococcus aureus*, *Escherichia coli*, *E. coli* O157:H7 and *Bacillus cereus* (bacterial strains) and *Penicillum* sp., *Saccharomyces cerevisiae*, *Candida tropicans* and *C. albicans* (fungal strains). The cultures were maintained freeze-dried. Bacterial strains were grown in Nutrient Agar whereas fungal strains were grown in Potato Dextrose Agar (PDA).

An overnight culture of each bacterial species grown in Nutrient Agar was diluted with fresh medium to achieve a final concentration of approximately 1-2 x 10^8 CFU/ml. The Agar Well Diffusion Method was performed for antibacterial activity. 15 ml of soft Nutrient Agar inoculated with 100 ml of overnight culture of each bacterial inoculum was spread uniformly onto pre-poured Nutrient Agar plate. The plates were left to get harden at room temperature for 15 min. and then 5 mm diameter holes were made in each agar gel and 100 µl control or 100 µM Al or 200 µM Al-treated *U. pilulifera* leaf extract was placed into each well. All plates were incubated for 24 h. at 37ºC under aerobic conditions. After incubation, bacterial growth was observed in each plate.

Also, the antibacterial and fungicidal activities of unexposed and exposed *U. pilulifera* leaf extracts against bacterial and fungal test strains was determined by disc diffusion method. 100 ml of broth from fresh culture of each bacterial or fungal inoculum (final concentration was approximately was 1-2 x 10^8 CFU/ml for each species) was inoculated into Nutrient Agar (used for bacterial test species) or Potato-dextrose Agar (used for fungal test species). Paper discs (Whatman no. 1, 6 mm) loaded with 15 ml solutions, prepared from controls or exposed groups were placed onto the surface of agar plates (Nutrient Agar was used for bacterial test strains whereas Potato-dextrose Agar was used for fungal strains). The plates were incubated at 37 and 24°C for 24 h. and 5-7 days for bacterial and fungal test species, respectively. In order to understand whether source of antibacterial and antifungal activities of *U. pilulifera* are from the compounds isolated from leaves of *U. pilulifera* exposed to Al stress or Al itself accumulated in leaves of *U. pilulifera* following application of Al stress, a control group (water + 11.31 mg l^{-1} Al) was prepared and applied on microorganisms used in this study. In control group, 11.31 mg kg^{-1} Al was used because leaves of *U. pilulifera* exposed to 200 µM $AlCl_3$ showed

11.31 ± 0.22 mg kg^{-1} Al accumulations. Therefore, an $AlCl_3$ solution was prepared containing 11.31 mg l^{-1} Al and antibacterial and antifungal effects of this solution was tested on microorganisms used in this study by employing the techniques of agar well and disc diffusion. After incubation, all plates were checked for any growth inhibition.

RESULTS AND DISCUSSION

Table 1 shows Al concentrations in roots and leaves of *U. pilulifera* grown in different treatment levels of Al. The concentrations of Al in *U. pilulifera* were increased dramatically when the application levels of Al increased. There was a large difference in Al concentrations among the roots and leaves of *U. pilulifera*. The concentrations of Al were increased significantly in roots at both application levels of Al and there was no Al accumulation at the application level of 100 µM Al whereas Al accumulation was seen at the application level of 200 µM Al in leaves (Table 1). In terms of Al accumulation capabilities, the roots and leaves of *U. pilulifera* showed very large differences, mainly accumulated in roots and only small amounts transported into the leaves.

TABLE 1
The concentrations of Al (mg kg^{-1} DW) in leaf and root parts of *U. pilulifera* grown in different treatment levels of Al (0, 100, and 200 µM) for two months.

	Plant Part	Control	100 µM	200 µM
Al (mg kg^{-1})	Leaf	0.00± 0.00	0.00± 0.00	11.31± 0.22*
	Root	0.00± 0.00	1643.3± 45.1*	8276.67 ± 64.7*

* Variance analysis and Tukey test are indicated (*$p < 0.05$ significant).

The antibacterial and antifungal activities of *U. pilulifera* leaf extracts were tested against nine strains of bacteria (5) and fungus (4) after the applications of Al stress. The antibacterial and antifungal activities of the leaf extracts showed varying magnitudes of inhibition patterns with standard positive control depending on the Al treatment levels. The inhibitory zones of control and 100 and 200 µM Al treated *U. pilulifera* leaf extracts against nine bacterial and fungal species were shown in Tables 2 and 3.

While analysis of 100 and 200 µM Al treated of *U. pilulifera* leaf extracts showed a low level of inhibition against bacterial species of *E. coli*, *Salmonella* sp., *Staphylococcus aureus* and *Bacillus cereus*, there was no inhibition against *E. coli* O157:H7 using the agar well and disc diffusion methods (Table 2). Also, untreated leaf extracts (control) showed no capacity to reduce or inhibit the growth of the *E. coli*, *Salmonella* sp., *S. aureus*, *B. cereus* and *E. coli* O157:H7 (Table 2). In our study, although extracts prepared from untreated *U. piluifera* leaves (control) did not exhibit any antibacterial activity against bacterial test species, the members of *Urticaceae* have been reported in the literature to have antibacterial activity against bacterial strains of *E. coli*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae*, *A. baumannii*, *S. aureus*, *B. subtilis*, *E. faecalis*, *S. enteritidis*, *S. gallinarum*, *S. agalactiae*, *S. typhi*, *Shigella flexneri*, and *S. sonnei* [5-6-8]. Concerning the antibacterial activity on test organisms in control groups may suggest that biologically active compounds were very low. Because of reduced growth caused by $AlCl_3$ (especially at the level of 200 µM treatment), only 5 gr of leaves were obtained from exposed groups and although more than 5 gr of leaves was obtained from control groups, in all antimicrobial activity experiments, equal amounts of leaves were used. Perhaps this may have prevented the emergence of antibacterial effect in control groups (dose-dependent). On the other hand, although the analysis of control, 100 and 200 µM Al treated leaf extracts showed a high level of inhibition capacity against *S. cerevisiae*, *C. albicans*, *C. tropicans* and *Penicillium* sp., 100 and 200 µM Al treated leaf extracts exhibited maximum inhibitory activity in comparison to the controls (Table 3). Our results from the controls were in accordance with the literature data [5-7-9]. In general, control and Al-treated *U. pilulifera* leaf extracts seems to be more potent showing a higher degree of antifungal activity in comparison to antibacterial activity (Table 3).

TABLE 2
Antibacterial activity of *U. pilulifera* extracts against bacterial species tested by disc diffusion and agar well assays.

Bacterial Species	Controls (0 µM $AlCl_3$ treated leaf extracts)		100 µM $AlCl_3$ treated leaf extracts		200 µM $AlCl_3$ treated leaf extracts		Water + 11.31 mg l^{-1} Al	
	Agar Well	Disc Diffusion	Agar Well	Disc Diffusion	Agar Well	Disc Diffusion	Agar Well	Disc Diffusion
Bacillus cereus	–	–	+–	+–	+–	+–	–	–
Escherichia coli	–	–	+–	+–	–	–	–	–
Escherichia coli O157:H7	–	–	–	–	–	–	–	–
Salmonella sp.	–	–	+–	+–	+–	+–	–	–
Staphylococcus aureus	–	–	+–	+–	+–	+–	–	–

TABLE 3
Antifungal activity of *U. pilulifera* extracts against fungal species tested by disc diffusion assay.

Fungal Species	Controls (0 µM $AlCl_3$ treated leaf extracts) Disc Diffusion	100 µM $AlCl_3$ treated leaf extracts Disc Diffusion	200 µM $AlCl_3$ treated leaf extracts Disc Diffusion	Water +11.31 mg l^{-1} Al Disc Diffusion
Candida albicans	+	+	+ +	–
Candida tropicans	+	+ +	+ +	–
Penicillum sp.	+	+ +	+ +	–
Saccharomyces cerevisiae	+	+ +	+ +	–

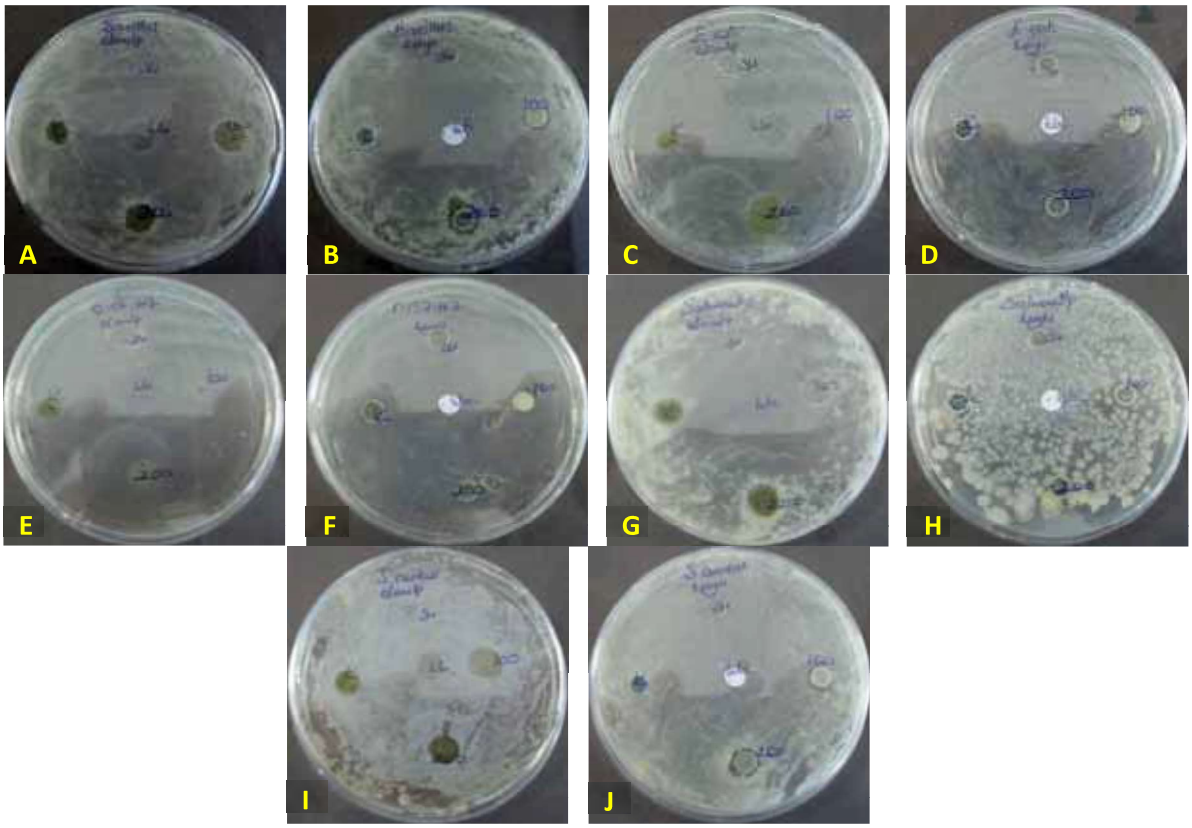

FIGURE 1
Antibacterial activity of *U. pilulifera* extracts against bacterial species tested by disc diffusion and agar well assays. A-*B. subtilis* agar well, B-*B. subtilis* disk diffusion, C-*E. coli* agar well, D-*E. coli* disk diffusion, E-*E. coli* O157:H7 agar well, F-*E. coli* O157:H7 disk diffusion, G-*S. typhi* agar well, H-*S. typhi* disk diffusion, I- *S. aureus* agar well, J- *S. aureus* disk diffusion.

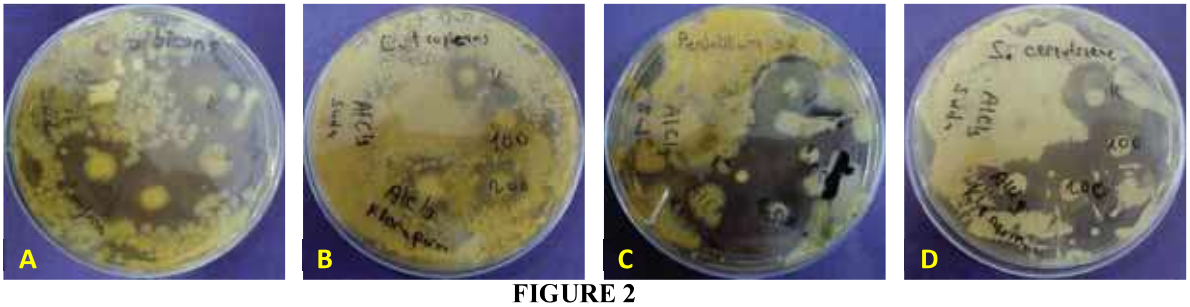

FIGURE 2
Antifungal activity of *U. pilulifera* extracts against fungal species tested by disc diffusion assay. A- *Candida albicans*, B- *C. tropicans*, C- *S. cerevisiae*, D- *Penicillum* sp.

As mentioned earlier, Al-treated leaf extracts exerted better antibacterial activity against bacterial species used in this study and no antibacterial activity was observed in controls. Although excessive amount of Al influences a large number of cellular processes negatively, including inhibition of K^+ [20], Ca^{2+} [21], and Mg^{2+} [22] uptakes, interaction with both microtubules and actin filaments leading

to disruption of cytoskeletal dynamics [23], modification of the cell wall and plasma membrane structures [24], alteration of phosphate and/or nucleotide metabolism [25], interfering with the signal transduction pathways [26] and causing oxidative stress [27], Al frequently stimulates growth at low concentration by alleviation of proton toxicity [28]. Also, previous studies showed that at least 30 genes are expressed in response to Al stress [29] and most of them seem to be general stress genes which are also expressed in response to pathogen infection [30], toxic heavy metals [31] or oxidative stress [32]. Moreover, the studies had been carried out for evaluating effects of heavy metals showed that secondary metabolite compositions were altered in plants. For example, the rates of phyllanthin and hypophyllanthin in *Phyllanthus amarus* Schum. and Thonn. and bacoside-A in *Bacopa monnieri* were altered by the applications of excessive levels of cadmium and iron, respectively [33]. In our study, it seems that although low concentration of Al (at 100 µM) application stimulates plant growth whereas high concentration of Al (at 200 µM) application inhibits plant growth. Because of increased plant growth or altered gene expression and secondary metabolite composition by the application of Al at low concentration, *U. pilulifera* seems to be gained better ability to synthesize substances such as phenolics and polyphenols, flavonoids, terpenoids, alkaloids and essential oils, lectins, tannins, polypeptides, serve as the components of plant defense mechanisms. The results showed that both agar-well and disc diffusion methods used in this study were equally effective for the detection of antibacterial activity on bacterial test strains (Table 2). Contrary to that, because of the changes at both cellular and whole plant level, growth arrest (and even death) was observed in response to Al toxicity at high concentration in *U. pilulifera*. Excessive Al causing toxicity symptoms due to a range of interactions at both cellular and plant levels stimulates the formation of free radicals and reactive oxygen species ending with in oxidative stress [28], inhibiting mineral uptake, metabolism and cytoskeletal dynamics [34], disrupting plasma membrane and the membrane transport processes [24]. Our data indicated that the antibacterial activity obtained after the applications of high levels of Al against five bacterial test strains was slightly different than the antibacterial activity obtained after the applications of low levels (the only difference was that after the application of Al stress at low concentration antibacterial activity was noticed whereas there was no activity after the application of Al stress at high concentration against *E. coli*). Although no antibacterial activity was recorded after the application of Al stress at high concentration, there was antibacterial activity after the application of Al stress at low concentration against *E. coli* (Table 2). The reason is because of negative effects of Al mentioned above. On the other hand, although controls and Al-treated *U. pilulifera* leaf extracts (at 100 and 200 µM) showed a high level of antifungal activity, Al-treated leaf extracts had better capacity for inhibition of fungal growth (Table 3).

CONCLUSIONS

The evaluations were done in terms of the antibacterial and antifungal activities of *U. pilulifera* leaf extracts against nine strains of bacteria and fungus after the applications of different levels of Al exposures. The reason for better antibacterial and antifungal activities could not be accumulated $AlCl_3$ itself in *U. pilulifera*. Because no antibacterial and antifungal activities were recorded after the applications of Al treatments alone (Tables 2 and 3). In our experiments, water + 11.31 mg l^{-1} Al was used for understanding of inhibitory antibacterial and antifungal effects of Al (if any) on microorganisms used in this study. Also, it was noticed that especially antifungal activity of *U. pilulifera* was increased substantially after the applications of different levels of Al exposures. It implies that Al had effects on plant growth and changed the expression of genes related with Al stress and secondary metabolite composition in cells and subsequently Al uptake had positive effects at low concentration and negative effects at high concentration on the antibacterial and antifungal activities of *U. pilulifera*.

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