International conference ON MODERN RESEARCH IN AGRICULTURAL SCIENCE AND ENVIRONMENT December 15, 2015 MALAYSIA – KUALA LUMPUR

کنفرانس بین المللی پژوهش های نوین در علوم کشاورزی و محیط زیست ۲۴ آذر ماه ۱۳۹۴ کوالالامپور - مالزی

Antibacterial activity of crude ethanolic, methanolic and ethyl acetate of *plantago ovata* affected by salt stress

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Abstract

As far as, salinity stress is the major factor affecting plant production, in this research, theeffect of different salinity stress levels (0 as a control, 25, 50, 100, 200 and 300 mM NaCl) on some of the biochemical parameters of *P. ovata* ethanolic extract were evaluated for 30 days. Furthermore, the antibacterial activity of ethanolic, methanolic and ethyl acetate extracts of *P. ovata* samples were determined in each treatment. The results indicated a significant increasing in proline, total flavonoids and total saponins with the enlargement of NaCl in the medium. Moreover the antibacterial activity of *P. ovata* extracts was increased with increasing salt level in the medium. The increased antibacterial activity with increasing NaCl treatments, suggest that the secondary metabolites are effective on *P.ovata's* resistance to salinity stress. It seems these secondary metabolites increase antibacterial activity of *P. ovata* and protects it from ion-induced oxidative stress, probably due to a common structural skeleton and the phenyl group of those metabolites. Collectively, our results indicate that *P. ovata* has physiological traits associated with resistance to salinity through increasing antibacterial activity and accumulation of secondary metabolites which made it as useful plant for growingin saline contaminated sites.

Keywords: Antibacterial activities, *Plantago ovata*, Salt stress, Salt tolerance, Secondary metabolites.

Introduction

Concern has been expressed about the rising prevalence of pathogenic microorganisms, which are resistant to the newer or modern antibiotics that have been produced in the last three decades (**Cohen, 1992., Gislene et al, 2000., Valarmathy et al, 2010)**.Antibiotic resistance has long been a significant problem in treatment of bacterial infection since several types of bacteria have been found to be able to resist more than one type of antibiotics. This results in the need of higher dose use with increased risk of drug toxicity or consideration to change the regimen. Moreover, excessive budget is currently spent on import of antibiotics manufactured abroad (**Ongsakul et al, 2009)**.

For over thousands of years now, natural plants have been seen as a valuable source of medicinal agents with proven potential of treating infectious diseases and with lesser side effects compared to the synthetic drug agents(**Iwe et al, 1999)**. Natural antimicrobial compounds in plant spices were found to possess antimicrobial activity (**Shelef, 1983., Kim et al, 1995., Nanasombat and Lohasupthawee, 2005)**. Plants and plant products have been used extensively throughout history to treat medical problems (**AbuShanab et al, 2004)**.Herbal medicines have been important sources of products for the developing countries in treating common infectious diseases and overcome the problems of resistance and side effects of the currently available antimicrobial agents (**Kianbakht and Jahaniani, 2003., Oskay et al, 2009)**. They have multiple biological activities *in vitro* and *in vivo* such as antiadherence, antioxidant, anti-inflammatory and antibacterial. Antibacterial activity of local medicinal plants should be studied to provide alternative and locally available antibacterial regimens[4].Numerous studies have been carried out to extract various natural products for screening antimicrobial activity (**Nita et al, 2002., Ates and Erdogrul, 2003)**.

Plants produce a large variety of secondary products that their concentrations are strongly depending on the growing conditions and it is obvious that especially stress situations have a strong impact on the metabolic pathways responsible for the accumulation of the related natural products. In a series of experimental observations, it could be shown that plants which are exposed to salt stress produce a greater amount of secondary plant products such as phenols, terpenes as well as N and S containing substances such as alkaloids (**Harborne and Williams, 1992., Stewart et al, 2001., Winkel, 2002., Mosaleeyanon et al, 2005., Couceiro et al, 2006)**.

Plantago ovata (Isabgol) is an annual herb cultivated as a medicinal plant generally grown in India, Pakistan and Iran (**Khaliq, 2011)**. It has been used in medicines since ancient times, but it has only been cultivated as a medicinal plant in recent decades (**Handa and Kaul, 1999)**. Since, salt stress is one of the most serious factors limiting the productivity of different crops and especially quantity and quality of their metabolic (secondary plant products) products to a greater extent, It could be possible to enhance a wide variety of useful metabolites in plant through applying different salinity treatments (**Cisneros, 2003)**. It is assumed that accumulation of secondary metabolites enhances the *P. ovata* capacity for salt tolerance. Thus this research goal was to study the effect of salinity stress on the proline, flavonoids and saponin as secondary metabolites contentand relationship between these metabolites production and antibacterial activity of *P.ovata*.

Experimental

Plant material, growth conditions, and treatments: Mature seeds of *P. ovata* were sterilized in 70% ethanol for 1 min, 0.1% mercuric chloride for 5min, followed by three

washes in sterile distilled water. After sterilization, seeds were germinated into pots filled with perlite. The uniform seedlings were feeding with modified 10% Hoagland nutrient solution containing: 0.2 mM KH_2PO_4, 0.8 mM$Ca(NO_3)_2.4H_2O$, 1 mM KNO_3, 0.4 mM $MgSO_4.7H_2O$, 15 µM FeEDTA, 10 µM H_3BO_3, 3 µM $MnCl_2.4H_2O$, 0.2 µM $ZnSO_4.7H_2O$, 0.2 µM $CuSO_4.5H_2O$, 0.1 µM $Na_2.MoO_4.2H_2O$.

Plants were grown in growth room with 16/8 h light/dark cycles, day/night temperature of 26/20 ˚C and light intensity approx, 280 mmol m^{-2} s^{-1}. The nutrient solution was renewed every week. Two weeks later the solutions were amended with eight NaCl concentrations (0, 25, 50, 100, 200 and 300 mM) for another 4 weeks. Every third day the perlite was flashed with deionized water to prevent a potential toxic build up of nutrient salts in the substrate and plants were received 200 ml of the appropriate solution. At harvest, plants were divided into root and shoot fractions. Air dried samples were cut with stainless steel scissors, weighted and ground in mortar to obtain homogeneous samples.

Proline, flavonoid and saponin determination: Proline was quantified according to the method described by Bates et al. (1973). Shoot samples (0.1 g) from each group were homogenized in 3% (w/v) sulfosalicylic acid and then the homogenate was centrifuged. The mixture was heated at 100 ºC for 1 h in a water bath after the addition of acid ninhydrin and glacial acetic acid. One ml of supernatant was used to determine the proline content colorimetrically by Tomas 302 spectrophotometer (Kyoto, Japan).
The flavonoids content was determined with modified aluminum chloride colorimetric method as described by Woisky and Salatino (1998). The shoots of *P. ovate* were extracted with ethanol under reflux in water bath at 80 ºC and then filtered. 0.5 mL of ethanol extracts were separately mixed with mixture of 95% ethanol, 10% aluminum chloride, 1M potassium acetate and distilled water. After incubation at room temperature, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan).
For determination of total saponins content, the dry shoot powder was extracted using 80% ethanol, and supernatant was collected after twice centrifugation. To ethanol solution of sample, vanillin and sulfuric acid were added and mixed well in an ice water bath. The mixed was warmed in a bath at 60 ºCfor 10 min, and then cooled in ice- cooled water. The absorbance of the mixture was recorded against the blank at 450 to 700 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan).

Determination of antimicrobial activity
Plant extracts preparation: The plant extracts were prepared using the modified method of Alade&Irobi. Briefly, three 100 g portions of the dried powdered plant were soaked separately in 500 ml of ethanolic (95 %), methanolic (95%) and ethyl acetate (98%) extracts, for 72 h. Then, each mixture was refluxed followed by agitation at 200 rpm for 1 h. The filtrates obtained were concentrated under vacuum at 40°C to obtain the dry extracts.

Measurement of minimum inhibitory concentrations: Minimum inhibitory concentrations (MIC)were measured within the range of ½ (v/v) extractsto 1/1024(v/v), using tube assaymethod. Fresh bacterial suspensions were prepared in McFarland turbidity. For the tube assay, 200 λ (0.2 ml) of Mueller Hinton broth added to each tube. Finally, 0.02 ml of bacteria adjusted to MacFarland standard in Mueller Hintonbroth was added to the tubes.

Microorganisms: *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus areus was* obtained from the Microbiology Laboratory of microbiology lab of Razi University. Standard strains of microbes were used which was incubated at 37ºC for 24 h.

Results and Discussion

Most of *P. ovata* salinity treatments showed antimicrobial activity in regards to all microorganisms tested (Table 1, 2 and 3). Methanolic, ethanolic and ethyl acetate extracts of *P. ovata* growth in highest NaCl treatment (300 mM) presented the lowest MICs against *E. coli* (125 µg/ml) compared to *P.aeruginosa*(500 µg/ml).The ethanolic extracts presented higher activity as inhibition was detected applying 125 µg/ml of the extract in low NaCl treatments (50 mM) (Table 1).

Table 1: Minimum inhibitory concentrations (MIC) measured for 95% Ethanolic Extracts. Values are shown in µg/ml.

Microorganisms	salinity					
	0	25	75	125	200	300
Escherichia coli	250	250	250	125	125	125
pseudomonas aeruginosa	500	500	500	250	250	250
Bacillus subtilis	250	250	125	125	125	125
Staphylococcus areus	500	500	250	250	250	125

Methanolic extract was more effective against *S. areus* in comparisons with other studied extracts (Table 2). This extract did not present activity against *P. aeruginosa.* Ethyl acetate extracts presented low activity as inhibition was detected only when applying 500 µg/ml of the extract in (100 mM NaCl treatment) against most of studied micro-organisms (*S. areus*, *P. aeruginosa* and *E. coli*) (Table 3).

Table 2: Minimum inhibitory concentrations (MIC) measured for 6 methanolic Extracts. Values are shown in µg/ml.

Microorganisms	salinity					
	0	25	75	125	200	300
Escherichia coli	500	500	250	250	125	125
pseudomonas aeruginosa	500	500	500	500	500	500
Bacillus subtilis	1000	1000	1000	250	250	125
staphylococcus areus	500	500	250	125	125	125

This demonstrates that ethanol is the most adequate solvent for the extraction of compounds with microbial activity from the leaves of *P. ovata.*

Table 3:Minimum inhibitory concentrations (MIC) measured for 6 Ethylene Acetatic Extracts. Values are shown in µg/ml.

Microorganisms	salinity					
	0	25	75	125	200	300
Escherichia coli	1000	1000	500	500	250	125
pseudomonas aeruginosa	1000	500	500	500	250	250
Bacillus subtilis	500	500	250	250	250	125
staphylococcus areus	1000	1000	500	500	250	250

The lack of antibacterial activity in the compounds separated by different extraction methods could be related to their low concentration after fractionation or due to possible synergism, that is - compounds do not present or present lower activity when separated into its isolated form. Such synergistic effect has been observed also in *Hypericum perforatum* in which the inhibitory affects was detected only in pooled fractions from leaves (**Oliveira et al, 2012)**
In some cases, the three extracts of *P. ovata* had antimicrobial activity against the same microorganism. For instance, the three extracts were active against such as *E. coli* and *B. subtilis*. This possibly means that the compound responsible for the antimicrobial activity was present in each extract at a different concentration (**Oyedeji et al, 2005)**.

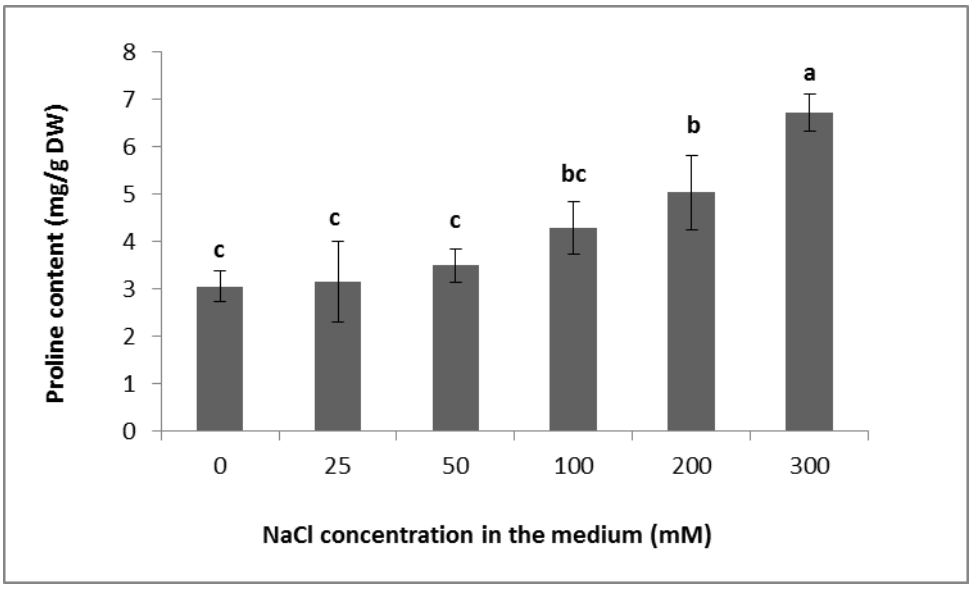

Figure 1: Changes in proline concentration of *Plantago ovata* Forsk. as affected by NaCl induced stress after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent ± standard error.

The *P. ovata* exhibited different levels of secondarymetabolites under salinity stress(Fig 1, 2 and 3). Generally, plant secondary metabolism and its metabolites result from the response and adaptation to different environmental stresses during long process of evolution, and hence, the production of secondary metabolites closely relate to environmental factors (including biotic and abiotic factors). These compounds could be responsible for its antimicrobial activity against *E. coli, B. subtilis, P. aeruginosa, and S. aureus.* The increased antibacterial activity with increasing NaCl treatments, suggest that the secondary metabolites are responsible to *P. ovata* salt tolerance. It seems these secondary metabolites increase *P. ovata* antibacterial activity and protects it from ion-induced oxidative stress, probably due to a common structural skeleton and the phenyl group of those metabolites.Flavonoids are known to be synthesized by plants in response to abiotic stress and microbial infection (**Dixon et al, 1983)** and are effective anti-microbial substances against a wide array of micro-organisms. Saponins are triterpenoid and a type of secondary metabolites that are produced by plant species like *P. ovate* (Fig, 3). The natural role of saponins in plants is likely to be in defense against pathogens (**Shi et al, 2004)**. Proline acts as an osmoticum, a protective agent of enzyme and cellular structure and a storage compound of reducing nitrogen for rapid regrowth after stress are relieved (**Misra et al, 2004)**. It was also approved that proline could react with hydroxyl radicals thereby protecting lipids, DNA, proteins and macromolecular structure from degridative reactions leading to cell destructions during salinity stress (**Khaliq et al, 2011)**.

A wide variety of flavonoids, saponins and proline products from *P. ovata*, can be useful as anti-microbial. The activity is probablydue to their ability to form a complex with extra-cellularand soluble proteins, which then binds to bacterial cellwall. More lipophilic components may also disruptmicrobial membranes (**Tsuchiya et al, 1996)**. Secondary metabolites lacking hydroxylgroups on their b-rings are more active against micro-organisms and the microbial target is the membrane with–OH groups (**Chaurasia and Vyas, 1997)**.

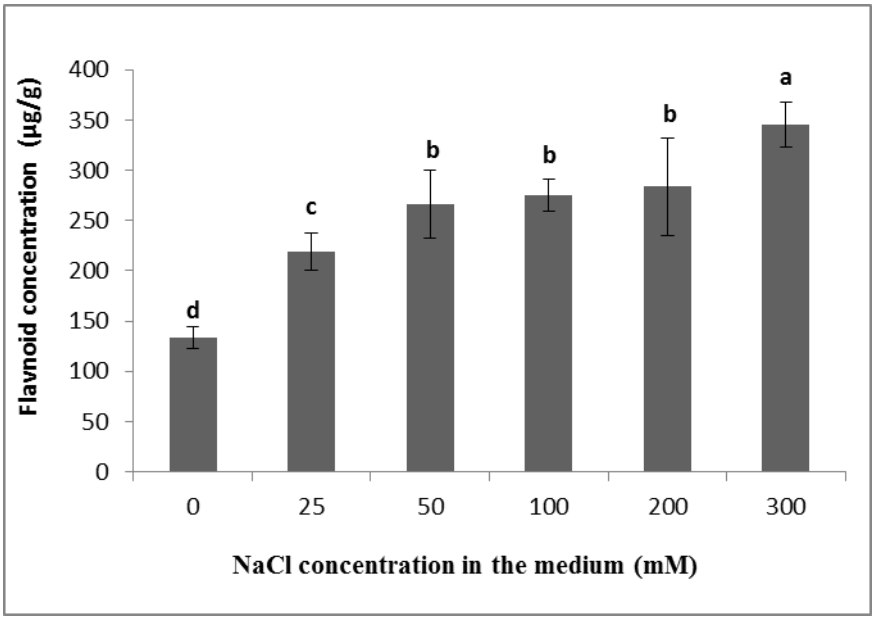

Figure 2: Effect of NaCl induced stress on flavonoids concentration of *Plantag oovata* Forsk. after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent ± standard error.

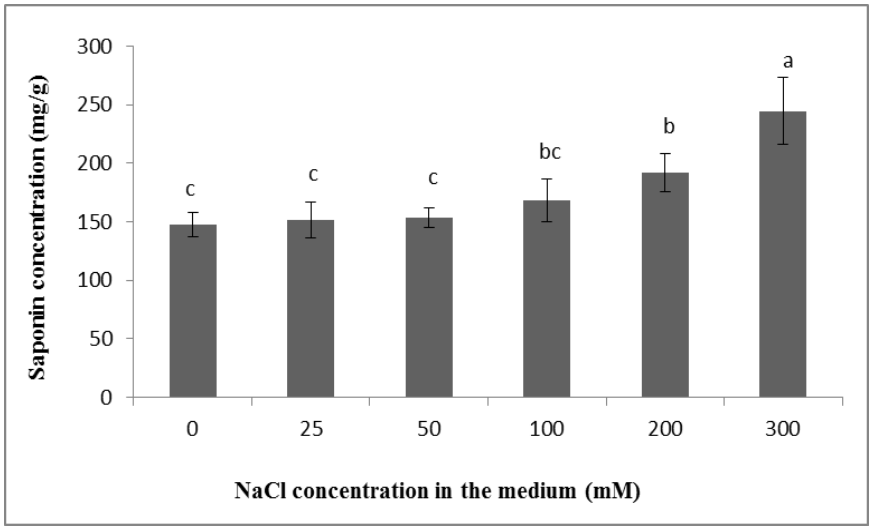

Figure 3: Effect of NaCl induced stress on saponin concentration of *Plantago ovata* Forsk. after 4 weeks of exposure to stress. Each value is the mean of three replicates and vertical bars represent ± standard error.

Conclusion: All the extracts showed various degrees of antimicrobial activity on the microorganisms tested. Some of these plants were more effective in the compare of traditional antibiotics to combat the pathogenic microorganisms. The chance to find antimicrobial activity was more apparent in ethanol than another extracts of the same treatments. A significant increase in proline, total flavonoids and total saponins with the enlargement of NaCl in the medium were found. Moreover the antibacterial activity of *P. ovata* extracts was increased with increasing salt level in the medium. The increased antibacterial activity by increasing NaCl treatments, suggest that the secondary metabolites are effective on *P.ovata*antibacterial activity. The results indicated that *P. ovata* could be a source of new antibiotic compounds. Further work is needed to isolate the secondary metabolites from the extracts studied in order to test specific antimicrobial activity.

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