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Research Article

Salt-induced alternations in the content of secondary metabolites and antioxidant responses of guava seedlings

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Abstract

Guava (*Psidium guajava* L.) is adapted to an extensive range of soils. However, its development is strongly affected by salinity. Environmental stresses are of the major and influential factors in the amount of plant secondary metabolites, especially phenolic compounds. The purpose of this investigation was to study the leaf antioxidant and flavonoid contents (catechin, quercetin and rutin) of guava seedlings grown under 0 and 100 mM of NaCl treatment. Leaf samples collected 12 weeks after salinity stress treatment and total phenol, antioxidant capacity, malondialdehyde, catalase, peroxidase and protease activities and high performance liquid chromatography were examined. The research was conducted in a completely randomized block design and Duncan test was used for mean comparison. According to the results, salinity enhanced total phenol content amount 2.09 times. The MDA value improved in guava leaves from 0.42 mg g^{-1} FW under non-stress conditions to 0.76 mg g^{1} FW under salinity. The most catalase (182.11 µmol min^{-1} g^{-1} FW), protease (9.70 µmol min^{-1} g^{-1} FW) and peroxidase (19.99 µmol min^{-1} g^{-1} FW) activities were observed in salt exposed plants. The antioxidant capacity of the leaf extract was 62.90% in the control plants, which enhanced to 77.46% after salt treatment. The most abundant flavonoid composition of the leaf extract was rutin (770 µg mg^{-1} DW) in the control treatment and catechin (1110 µg mg^{-1} DW) in salt exposed plants. We concluded that salinity stress, in addition to a noticeable increase in the activities of catalase, peroxidase and protease enzymes, enhanced phenol, catechin and quercetin contents.

Key words: Antioxidant capacity, Catalase, Catechin, Malondialdehyde, Peroxidase, Total phenol

Introduction

Salinity, as one of the restricting factors of plant growth and production in arid and semi-arid regions (Kaashyap *et al*., 2018), affected approximately 30% of agricultural lands (due to low rainfall, high surface evaporation, irrigation with saline water and numerous cultivation activities) (Machado and Serralheiro, 2017).

Salinity stress leads to the accumulation of free radicals and oxidation of proteins and lipids (Molassiotis *et al*., 2006a; Gill and Tuteja, 2010). It affects plant physiology through osmotic stress, toxicity of Na^+ and Cl^-, stomatal closure and declined photosynthesis (Munns and Tester, 2008).

Guava (*Psidium guajava* L.), a member of the Myrtaceae family (Gutierrez *et al*., 2008), is rich in tannins, triterpenes, quercetin flavonoids, guajanoic acid, saponins, carotenoids, leucocyanidin, elajic acid, beta-cystosterol (Jimenez-Escrig *et al*., 2001). Also, the high nutritional value of guava is related to its fiber, vitamin A, vitamin C, folic acid, potassium, copper and manganese contents (Joseph and Priya, 2011; Qin *et al*., 2017).

Guava leaf extract possesses analgesic, anti-inflammatory, anti-microbial and antioxidant activities (Ryu *et al*., 2012; Roy *et al*., 2006; Nair and Chanda, 2007; Chen and Yen, 2007). Accordingly, gallic acid, catechin, epicatechin, rutin, naringenin and kaempferol have been found in guava leaves (Wu *et al*., 2008; Melo *et al*., 2011; Chen and Yen, 2007).

Guava is adapted to an extensive range of soils, but its development is strongly affected by salinity (Cavalcante *et al*., 2005). Reportedly, water salinity, above 1.75 dS m^{-1} has reduced the quality and growth of guava seedlings (Cavalcante *et al*., 2010). The main impacts of salinity on guava have been indicated as a reduction in leaf number (Sa *et al*., 2016), shoot/root (Souza *et al*., 2017), stem diameter (Silva *et al*., 2017), root dry weight (Gurgel *et al*., 2007), photosynthetic efficiency (Ebert *et al*., 2002), leaf chlorophyll content and the activity of antioxidant enzymes (Ali-Dinar *et al*., 1999).

The generation of reactive oxygen species (ROSs), including superoxide (O^-), hydroxyl (OH^-) and hydrogen peroxide (H_2O_2), which occurs in stress exposed-plants, leads to degradation of membranes, lipids, proteins and nucleic acids (Bailly, 2004; Garratt

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et al., 2002). Under such conditions, antioxidant mechanism is activated to protect cells via defense antioxidant enzymes (such as phenylalanine ammonialyase) and non-enzymatic antioxidants (like phenylpropanoid compounds) (Agarwal and Pandy, 2004; Bettaieb *et al*., 2011).

Also, environmental stresses are influential factors in the amount of plants secondary metabolites. There are evidences of increased secondary metabolites under environmental stress. However, some studies have indicated that this impact is not permanent and even reduced content has stated (Ramakrishna and Ravishankar, 2011; Walpola and Arunakumara, 2017).

Plant phenols are a chemically heterogeneous group of approximately 10000 detached compounds. Some are soluble in organic solvent, water, carboxylic acids or glycosides, and the rest are insoluble (Taiz and Ziger, 2002). These compounds play role in scavenging of free radicals and reactive oxygen species under abiotic stresses (Dicko *et al*., 2005). They protect structural proteins, enzymes, nucleic acids, lipoproteins and membrane lipids (Durazzo and Lucarini, 2019; Durazzo *et al*., 2019; Karaaslan *et al*., 2011). Enhanced synthesis of phenolic compounds has reported in plants under salinity stress (Al-Amier and Craker, 2006; Adil *et al*., 2007).

Flavonoids, a group of phenolic compounds, show protective activities under abiotic stresses, wounds, UV radiation, ozone exposure, contaminants and herbivores (Agati *et al*., 2012). The expression of antioxidant genes (Vinyard *et al*., 2005) and the phenyl-propanoid pathway (especially the flavonoid biosynthesis), develops under oxidative stress in plants (Mackerness *et al*., 2001; Watkinson *et al*., 2006).

Salinity stress seems to stimulate and improve the production of secondary metabolites by activating cellular mechanisms. Given the importance of guava in the southern regions of Iran, its widespread cultivation in Hormozgan province, the lack of suitable water and soil resources as well as the effect of environmental stresses on the amount of antioxidant compounds and secondary metabolites, the aim of the present investigation was to evaluate the antioxidant reaction and flavonoid content (Catechin, quercetin and rutin) of guava seedlings (grown under normal growth conditions or exposed to salinity stress through irrigation water) and to understand its mechanism.

Material and methods

Plant materials and experimental design: The present study was conducted at the nursery of the Horticulture Department, Faculty of Agriculture and Natural Resources, University of Hormozgan, Hormozgan, Iran (57° 4 E 27° 9 N, Elevation: 10 m, RH: 70%, Mean temperature: 24 ± 1 °C) during 2021.

The experiment was carried out in a completely randomized block design with six replications. The treatment was salinity with NaCl salt (0 and 100 mM). The applied sodium chloride was from the Merck Company, Germany.

One-year-old guava seedlings with a height of approximately 75 cm were prepared from a nursery located in Rudan city (Hormozgan province) and transferred to the Faculty of Agriculture research nursery and then cultivated. Simultaneous with soil preparation, 0.113 g of urea, 0.113 g of sulfate-potassium and 0.110 g of superphosphate were combined with each 5 kg of the soil mixture.

Salinity stress was applied through irrigation water from the beginning of the 5th week (after the establishment of seedlings) until the end of the 12th week. In order to prevent sudden stress, salinity treatment was applied gradually, as initiated with 25 mM and reached the final concentration in the following weeks. The treatment was applied with irrigation water until the end of the 12th week and then all plants were irrigated with distilled water for 4 weeks. Sixteen weeks after the establishment of seedlings (12 weeks after salinity stress treatment), leaf samples were collected to measure the following traits.

Total phenol: Leaf sample (0.1 g) was homogenized with 2 ml of 80% methanol and centrifuged (10000 rpm, 10 min). Distilled water (490 µl) and folin ciocalteu (500 µl) were added to 10 µl of the supernatant and incubated under dark conditions for 3 minutes. Afterward, 500 µl of 1% sodium carbonate was added and re-incubated under same conditions for 30 minutes more. Finally, the optical absorption of the extract was recorded by spectrophotometer (Model CE 2501 Cecil UK) at 765 nm and the total phenol content was expressed in mg g^{-1} gallic acid (Spanos and Wrolstad, 1990).

Antioxidant capacity (DPPH): The antioxidant capacity of guava leaves was determined using 2, 2-diphenyl-1-picryl hydrazyl. In order to evaluate the antioxidant capacity, 0.5 g of leaf sample was homogenized with 15 ml of 80% methanol and incubated under dark conditions (24° C, 24 h). The extract was then centrifuged (10000 rpm, 10 minutes). Subsequently, 40 µl of 80% methanol, 350 µl of 0.1 N 2, 2 diphenyl-1-picryl hydrazyl (DPPH) (containing 0.0394 g of DPPH and 100 ml of methanol) and 1.55 ml of methanol were added to 60 µl of the supernatant and re-incubated under dark conditions (4° C, 20 minutes). Finally, the optical absorption of the extract was recorded at 517 nm (Brand-Williams *et al*., 1995).

Malondialdehyde (MDA): The measurement of MDA was carried out according to Heath and Packer (1968) procedure. Briefly, 0.2 g of fresh leaf sample was homogenized with 5 ml of 0.1% TCA (Trichloroacetic acid), then centrifuged (10000 rpm, 5 minutes). Afterwards, 4 ml of 20% TCA solution containing 0.5% thiobarbituric acid (TBA) was added and then incubated (water bath, 95° C) for 30 minutes. The samples were then immediately cooled in ice and re-centrifuged (10000 rpm, 10 minutes). The absorption was recorded at 532 and 600 nm. The extinction coefficient was 155 $mM^{-1}cm^{-1}$. The malondialdehyde

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content was expressed in mg g^{-1}FW.

Preparation of the enzyme extract: In order to prepare the enzyme extract, 0.5 g of fresh leaf was ground with liquid nitrogen and homogenized with 1 ml of potassium phosphate extraction buffer (containing 50 mM potassium phosphate buffer, 0.0372 g of EDTA and 1 g of PVP). The extract was then centrifuged (15000 rpm, 15 minutes). The supernatant was utilized for the following enzyme assays (Dhindsa *et al*., 1981).

Catalase assay: Catalase activity was measured according to Dhindsa *et al*. (1981). Briefly, 50 µl of the enzyme extract was mixed with 1 ml of catalase reaction solution (including 50 mM phosphate-phosphate buffer and 15 mM hydrogen peroxide). Then the absorbance was measured with at 240 nm. The extinction coefficient is equal to 39.4 $mM^{-1}Cm^{-1}$.

Peroxidase assay: Peroxidase activity was measured by the method of Chance and Maehly (1995). The enzyme extract (33 µl) was mixed with 1 ml of peroxidase solution (containing 13 mM guaiacol, 5 mM hydrogen peroxide and 50 mM potassium phosphate buffer, pH=7). The absorbance was then measured at 470 nm after 1 min. The extinction coefficient is equal to 26.6 $mM^{-1}Cm^{-1}$.

Protease assay: In order to measure protease activity, 50 µl of the enzyme extract was incubated with 350 µl of 50 mM sodium phosphate buffer (0.44 g of sodium phosphate, 50 ml of distilled water and 400 µl of 1% casein, pH = 7.5) under laboratory conditions (24°C, 10 minutes). Afterwards, 800 µl of TCA (10%) was added and re-incubated for 20 min. After centrifuging (10000 rpm, 5 minutes), the optical absorption was recorded at 280 nm and then expressed in µmol min^{-1} g^{-1} FW. The extinction coefficient was 26.40 $mM^{-1}Cm^{-1}$ (Homaei and Samari, 2017).

High performance liquid chromatography (HPLC): The guava leaves were detached, cleansed, dried and powdered with an electric mill (under room temperature). To prepare ethanolic extract, 20 ml of 80% ethanol (HPLC grade) was added to 2 g of leaf powder and then shook (24° C, 110 rpm, 72 h) (shaker incubator Wise Cube model). The centrifugation (6000 rpm, 10 minutes) was followed by filtering the sample (Whatman filter paper). The ethanol was then removed using a rotary evaporator (45° C). Afterwards, the solution was dried in an oven (40° C). Finally, the extract was collected and stored in dark glass (-20° C until the usage) (Farhoosh *et al*., 2004).

Hurst *et al*. (1983) method was applied to perform HPLC. Firstly, the UV detector was heated for 10 minutes and the mobile phase was degassed through the separation column under experimental conditions for 20 minutes before injecting samples. Then, 20 µl of samples were injected. Isolation was performed at 280 nm at laboratory temperature. In order to prepare the standard sample, 5 mg of quercetin, catechin and rutin were dissolved in 50 ml of methanol, and then diluted concentrations were prepared. Afterwards, the solution was passed through a 0.2 µl filter. 0.1 g of dried extracts were dissolved separately in 2 ml of methanol and prepared for injection by HPLC (AZURA model, KNAUER Germany) after filtration.

Data analysis: The research was conducted in a completely randomized block design and the data were analyzed using MSTATC software. Duncan test ($P<0.01$) was used for mean comparison. Figures were drawn via Excel.

Results and discussion

Total phenol: According to the results, salinity stress enhanced the amount of total phenol (Figure 1). Salinity increased total phenol content amount 2.09 times. Phenolics are carbon-rich compounds and known as a large group of plant secondary metabolites. These compounds are also considered as antioxidants and may play a role in scavenging free radicals and reactive oxygen species under abiotic stress conditions (Dicko *et al*., 2005). The phenylpropanoid pathway produces various phenolic compounds with defensive functions. Phenylalanine ammonia lyase is a key enzyme in this pathway that mediates the formation of trans-cinnamic acids (Solecka and Kacperska, 2003). Polyphenol biosynthesis positively associated with abiotic stresses (Hafsi *et al*., 2017; Hafsi *et al*., 2016; Vogt, 2010). Guava seems to effectively counteract with the adverse impacts of salinity stress via enhancing the leaf phenol content. The results of this study were consistent with those obtained by Sorkheh *et al*. (2012) on wild almonds.

Malondialdehyde (MDA): Based on the results, the impact of salinity was significant on malondialdehyde content. The MDA value improved in guava leaves from 0.415 mg g^{-1} FW under non-stress conditions to 0.755 mg g^{-1} FW under salinity (Figure 2).

Accumulation of free radicals in cells caused damages to membrane lipids, produces lipid radicals and accelerate lipid peroxidation (Ashraf, 2009). Lipid peroxidation leads to alternations in the selective permeability of membranes and the activity of membrane-bound enzymes (Liang *et al*., 2003). MDA was the main product of lipid peroxidation and considered as an indicator of the ROSs under oxidative stress (Hong *et al*., 2000). According to Shao *et al*. (2005), the MDA content was augmented under stress, which is in line with the results of this study. MDA value was also ascended with increasing salinity in *Bruguiera gymnorrhiza* (Parida and Das, 2005). Nasibi *et al*. (2013) reported a significant enhancement in the amount of MDA in pistachio seedlings under cold stress.

Catalase activity: The impact of salinity on catalase activity was significant. The highest value (182.114 µmol min^{-1} g^{-1} FW) was observed in 100 mM salinity compared to control (150.108 µmol min^{-1} g^{-1} FW) (Figure 3).

Catalase acted to disintegrate H_2O_2 and convert it into water and oxygen (Parida and Das, 2005). Reportedly, the results of enhanced catalase in guava

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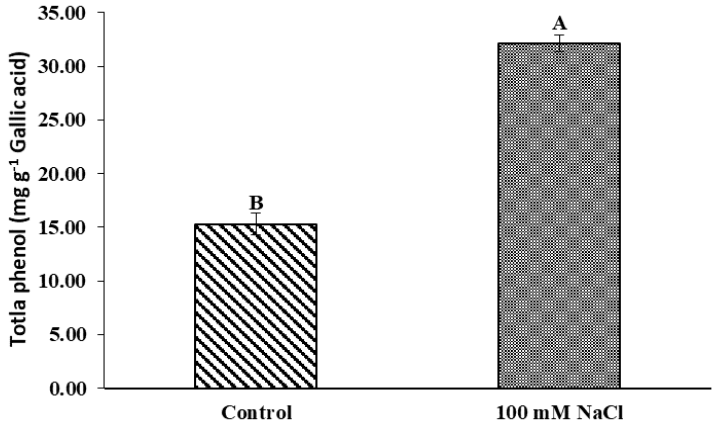

Fig. 1- The impact of irrigation water salinity on total phenol content of guava leaves. The same letters denote lack of statistically significant difference (Duncan test P<0.01)

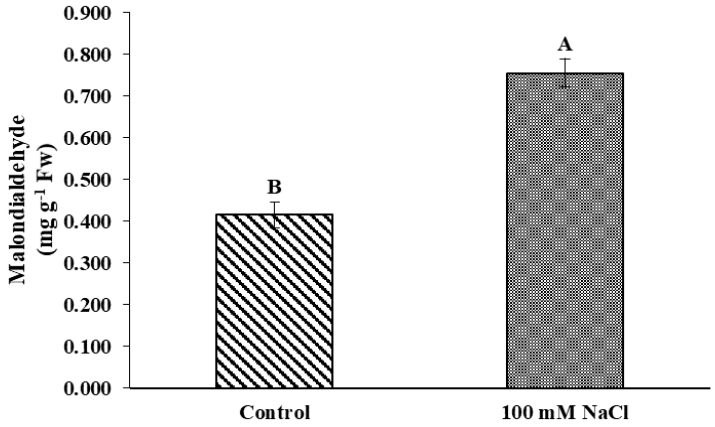

Fig. 2- The impact of irrigation water salinity on MDA value of guava leaves. The same letters denote lack of statistically significant difference (Duncan test P<0.01)

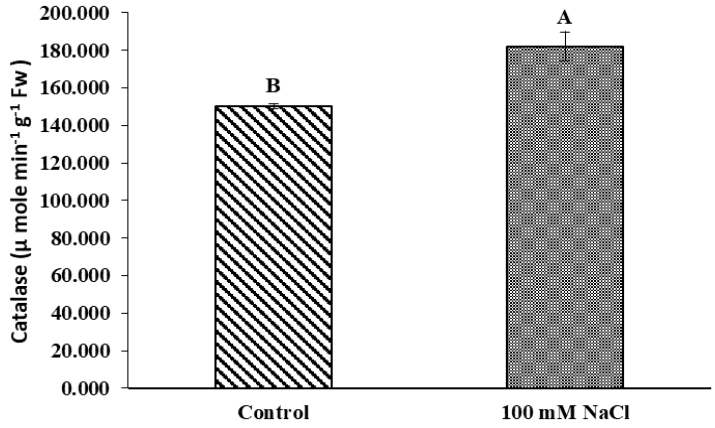

Fig. 3- The impact of irrigation water salinity on catalase activity of guava leaves. The same letters denote lack of statistically significant difference (Duncan test P<0.01)

(Esfandiari Ghalati *et al*., 2020), olive and citrus trees (Ben Ahmed *et al*., 2010; Balal *et al*., 2012), *Avicennia marina* (Takemura *et al*., 2002; Jithesh *et al*., 2006) under salinity stress, are in line with those of the current study.

Protease activity: In this study, salinity enhanced protease activity (Figure 4). The protease value was 7.23 µmol min^{-1} g^{-1} FW in the control and reached 9.70 µmol min^{-1} g^{-1} FW in 100 mM salt.

This enhancement was probably due to an increase in protein breakdown; The pathway included the generation of reactive oxygen radicals under salinity conditions, which leads to the oxidation of amino acid chain, formation of protein-protein bonds and ultimately

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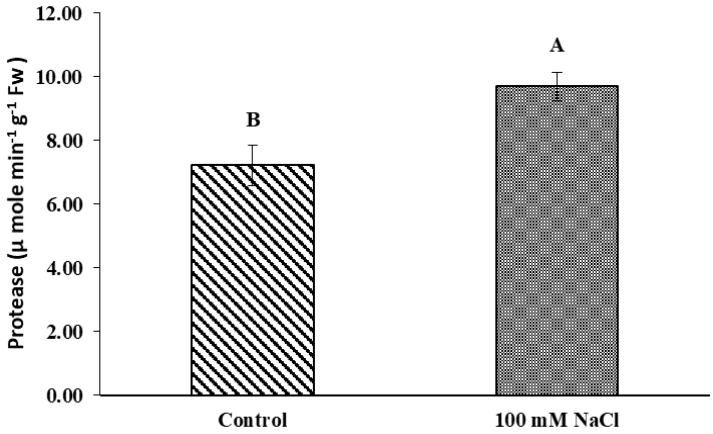

Fig. 4- The impact of irrigation water salinity on protease activity of guava leaves. The same letters denote lack of statistically significant difference (Duncan test P<0.01)

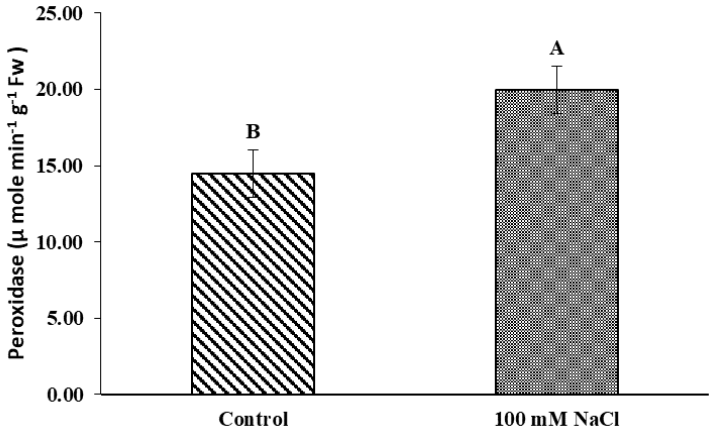

Fig. 5- The impact of irrigation water salinity on peroxidase activity of guava leaves. The same letters denote lack of statistically significant difference (Duncan test P<0.01)

increase protein breakdown (Bartels and Sunkar, 2005). There have been reports of enhanced protease activity under salinity stress (Hertwig *et al*., 1992; Parida and Das, 2005; Erturk *et al*., 2007).

Peroxidase activity: In the current assessment, the ascended peroxidase activity was observed in guava leaves under salinity and its value enhanced from 14.5 µmol min^{-1} g^{-1} FW (control) to 19.99 µmol min^{-1} g^{-1} FW (100 mM NaCl) (Figure 5).

Peroxidase accompanied by catalase regulate intracellular H_2O_2 levels (Willekens *et al*., 1995). Enhanced antioxidant enzymes reported in MM106 apple (Molassiotis *et al*., 2006b) and Gisela5 cherry rootstocks (Erturk *et al*., 2007) under salinity conditions. Increased peroxidase activity in salinity-adapted cells reflects alternations in the mechanical properties of cell wall, which may be associated with salt adaptation processes (Sancho *et al*., 1996). Some studies have also demonstrated an association between peroxidase activity and changes in cell wall and membrane integrity under salinity stress (Elsheery *et al*., 2020; Goharrizi *et al*., 2020; Lo'ay, and EL-Ezz, 2021).

Antioxidant capacity (DPPH): The antioxidant capacity of leaf extract was 62.90% in the control plants, which enhanced to 77.46% after salt treatment. DPPH radical scavenging activity, a measurement of non-enzymatic antioxidant activity (Kang and Saltveit, 2002), associated with the phenolic compounds contetnt (Ben Taarit *et al*., 2012; Huang *et al*., 2006; Joyce *et al*., 2005). Stresses increase the DPPH in cabbage (Sidsel Fiskaa *et al*., 2009) and orange (Klimczak *et al*., 2007), which are consistent with the results of the current study (Figure 6).

Flavonoid compounds assessment: The most abundant flavonoid composition of leaf extract was rutin (770 µg mg^{-1} DW) in the control treatment (table 1) and catechin (1110 µg mg^{-1} DW) in the salt treated plants (table 2). Among assessed compounds, quercetin had the longest inhibition time in both conditions and the last peak in the chromatogram of the mixture of standards was related to this compound (Figures 7 and 8).

About 72 phenolic compounds have been identified in guava leaves (Diaz-de-Cerio *et al*., 2016), quercetin was of the foremost ones (Kumar *et al*., 2021). High levels of catechin and epicatechin have also been reported in guava leaves. In addition, eugenol and iso-eugenol have been identified in guava leaves (Liu *et al*.,

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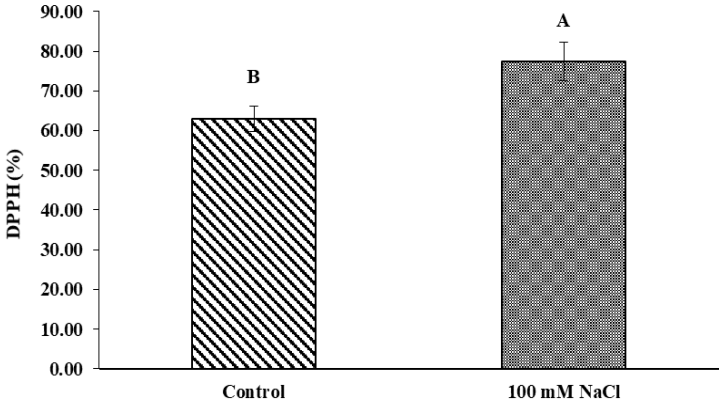

Fig. 6- The impact of irrigation water salinity on antioxidant capacity (DPPH) of guava leaves. The same letters denote lack of statistically significant difference (Duncan test P<0.01)

Table 1. Flavonoid compounds (µg mg^{-1} DW) in guava leaf extract under control conditions

Flavonoid component	Molecular formula	Inhibitory period (min)	Value (µg g^{-1} DW)
Catechin	$C_{15}H_{14}O_6$	3.967	370
Rutin	$C_{27}H_{30}O_{16}$	5.350	770
Quercetin	$C_{15}H_{10}O_7$	20.100	250

Table 2. Flavonoid compounds (µg mg^{-1} DW) in guava leaf extract irrigated with saline water

Flavonoid component	Molecular formula	Inhibitory period (min)	Value (µg g^{-1} DW)
Catechin	$C_{15}H_{14}O_6$	3.767	1110
Rutin	$C_{27}H_{30}O_{16}$	5.400	670
Quercetin	$C_{15}H_{10}O_7$	20.283	300

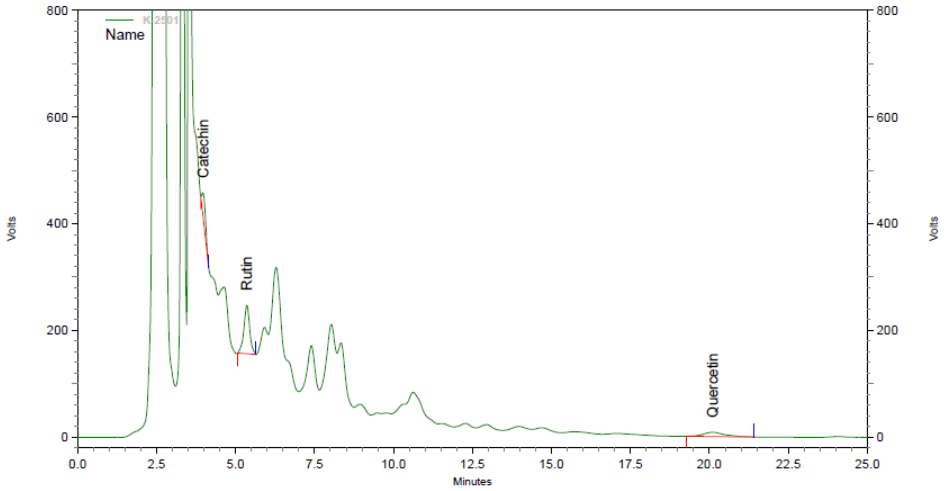

Fig. 7- Chromatogram of flavonoid compounds in leaf extract of guava plants irrigated by non-saline water.

2014). In the study of Chen and Yen (2007), 50 compounds in guava leaf essential oil were introduced using gas chromatography (GC) and gas chromatography / mass spectrometry (GC/MS), which beta-cariophylene, alpha Pinene and 1, 8 cineole were of the most important ones.

There are evidences regarding the multiplication of secondary metabolites under environmental stresses; However, some studies have indicated that this impact is not permanent and in some cases even declined amount of secondary metabolites is stated (Vickers *et al*., 2009; Ramakrishna and Ravishankar, 2011; Walpola and Arunakumara, 2017). Biosynthesis of phenylpropanoid pathway-derived compounds during environmental stresses is one of the evolutionary strategies of plants to confront stressful conditions, and flavonoids are a large group of compounds derived from this pathway (Ksouri *et al*., 2007). In the current study, the catechin content ascended significantly after exposure to salt. In fact, flavonoids are among the

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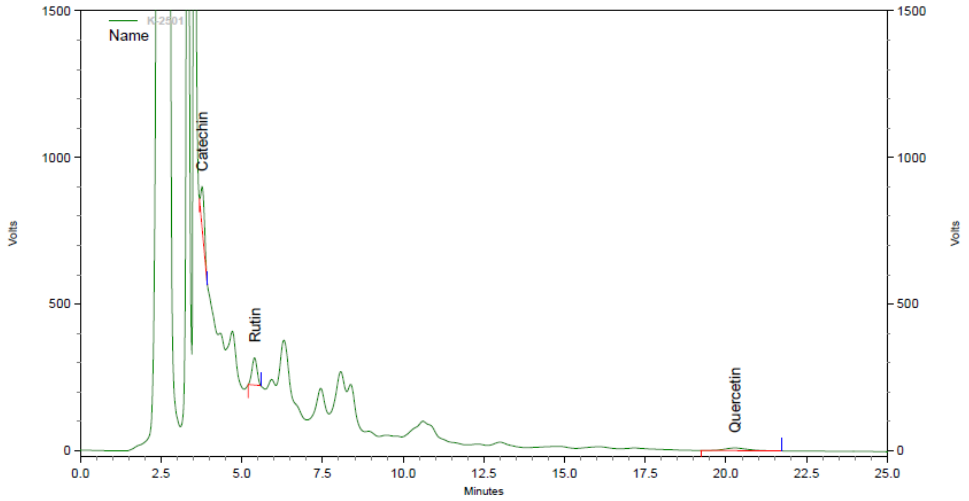

Fig. 8- Chromatogram of flavonoid compounds in leaf extract of guava plants irrigated by saline water.

antioxidants that plants produce to tolerate stressful conditions and thus respond to salinity stress.

Conclusion

In plants, biochemical and physiological responses, besides the synthesis of antioxidant compounds prevent oxidative stress damage and preserve them against reactive oxygen species. ROS detoxification system in plants includes non-enzymatic and enzymatic pathways. According to the results of this study, the contents of phenols and flavonoids (catechin, quercetin) were increased significantly along with a noticeable improvement in the enzymatic system (catalase, peroxidase and protease activities), which eventually led to enhanced antioxidant capacity under revealed stress.

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