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Research Article

EFFECT OF SALICYLIC ACID PRETREATMENT ON KHAT LEAVES RESIDUES IN WHEAT LEAVES

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ABSTRACT
The interactive effects khat leaves residues and salicylic acids (SA) were studied in wheat leaves. Addition of khat leaves residues into the soil lead to the reduction of chlorophylls **a** and **b**, ascorbate (AS), glutathione (GSH) and non-protein thiol (NPT) contents, catalase (CAT) and ascrobic peroxidase (APX) activities in wheat leaves. However, application of salicylic acid (SA) significantly increased chlorophylls **a** and **b**, (AS), (GSH), (NPT) contents, (CAT) and (APX) activities. Electrolyte leakage (EL%), peroxidase (POD) activity, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents were increased at the concentrations of khat leaves residues employed in this study. Soaking wheat caryopses in (SA) counteracted partially or completely the adverse effects of khat leaves residues on (EL%), (H_2O_2), (MAD) and (POD) activities.

Keywords: *Salicylic Acid, Khat, Wheat, Chorophylls, Antioxidants, Hydrogen Peroxide, Lipid Peroxidation*

INTRODUCTION
Allelopathy is a phenomenon of releasing allelochemical compounds into the environment from plants, has a variant effect which can be either direct or indirect, beneficial or adverse effects on the same kind or another plant. Allelochemicals may by present in all plant parts, leaves, stems, roots and seeds and the quantities vary from one tissue to another (Yu *et al.*, 2003 and Filemon *et al.*, 2013). In addition, researchers recorded that different types of abiotic and biotic stress can alter the production and release of allelochemical during the vital cycle of plants (Mahajan and Tueja, 2005; Al-Hakimi, 2008).
Salicylic acid (SA) as a phenolic plant compound used for internal regulator hormone, due to its defensive mechanism against biotic and abiotic stresses. Some earlier reports display that exogenous (SA) can ameliorate the impairing effects of drought (Al-Hakimi, 2006), salt stress (Al-Hakimi and Hamada, 2001) and khat leaves residues (Al-Hakimi, 2008). Khat (*Catha edulis* Forskal) belongs to the Celastraceae family, is a wild plant, cultivated in several countries including Yemen. People chewed the young leaves and shoots for that purpose, while the rest of the twigs are discarded.
The problem of khat in Yemen is a considerable area of agricultural lands that must be cultivated with several economic crops is currently replaced by khat plantation. The main objective of the present study was to investigate the interactive effects of (SA) and khat leaves residues on the alterations of some physiological parameters observed of khat leaves-stressed in wheat leaves.

MATERIALS AND METHODS
Plant Materials and Treatments
Caryopses of wheat (*Triticum aestivum* L.) were soaked for 6h in aerated water and 0.5mM (SA), separately before sowed in plastic pots (20cm diameter and 15cm depth) contained 2kg soil (clay and sand 2:1 by volume) and lined with polyethylene bags. Detached leaves from the twigs of khat (*Catha edulis* Forskal) were dried at 70ºC and then ground with an electric mill to provide the residues. The weight of khat powder (0, 25 and 50) gm/pot were mixed with about 5cm depth of soil in each pot. After that, caryopses were cultivated in each of 30 pots and irrigated with distilled water until the appearance of the first pair of true leaves. Three identical individuals were left in each pot and were irrigated regularly, the amount of water lost per day was compensated with distilled water to the level of field capacity (30%). Plants were allowed

to grow for 30 days under field conditions. The average prevailing climatic conditions were: temperature max. 26 ± 2 °C and min. 14±2 °C, relative humidity max. 58 ± 3% and min. 38 ±2%.

Chlorophyll Determination

The contents of chlorophyll **a** and **b** were extracted from 0.1gm fresh weight of leaves by 80% acetone. The content of chlorophylls were determined according to the procedure described by Porra, (2002).

Determination of Electrolyte Leakage (EL%)

Cutting 200mg fresh leaves into small pieces about 5mm size and placed them in to test tubes containing 10ml distilled deionized water. The tubes were covered with plastic caps and then placed in a water bath at 32°C. After 2h of incubation in the water bath, the initial electrical conductivity of the medium (EC1) was determined with an electrical conductivity meter. Thereafter, the samples were autoclaved at 121°C for 20 min to get released all electrolytes. The temperature of samples was brought down to 25°C and the final electrical conductivity (EC2) measured. The formula was (EL% = EC1/EC2 ×100) devised by Dionisio-Sese and Tobita, (1998).

Determination of Hydrogen Peroxide (H2O2)

Homogenized 0.5gm fresh leaves in an ice bath with 5ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenized was centrifuged at 12,000 rpm for 15min, 4ºC, and 200µl of the supernatant was added to 200µl of 100mM potassium phosphate buffer (pH 7.0) and 800µl of 1M potassium iodide (KI). The absorbance at 390nm according to He *et al.*, (2005). The content of (H_2O_2) for all samples was determined using (H_2O_2) as a standard.

Determination of Lipid Peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation according to the method described by Madhava Rao and Sresty, (2000) with some modifications as suggested by Weisany *et al*., (2012). Homogenized 0.5gm fresh leaves of wheat with 2.5ml of 0.1% (TCA) solution. The extract was centrifuged at 10,000 rpm for 10minutes. Then, added 4ml TCA (20%) solution containing 0.5% thiobarbituric acid (TBA) to every 1ml of the aliquot. After properly treating the mixture, it was centrifuged for 15minutes at 10,000 rpm, and the absorbance of the supernatant was measured at 532nm. The level of lipid peroxidation was expressed as µmol of (MAD) formed using an extinction coefficient of 155 mM^{-1} cm^{-1} as µmol g^{-1} fresh weight.

Determination of Antioxidant Enzyme Activity

The activities of (CAT) and (POD) were measured by Yang *et al*., (2010) with slight modifications. The (CAT) assay mixture 3ml contained 15mM sodium phosphate buffer (pH 7.0), 10 mM (H_2O_2), and 0.1ml of enzyme extract. The activity was measured at 240nm. The (POD) reaction mixture 3ml consisted of 10mM guaiacol, 50mM (H_2O_2), 0.2M phosphate buffer (pH 6.0) and 0.1ml of enzyme extract. The activity was measured at 470nm. The (APX) activity was determined according to Wang *et al*., (2008). The mixture 3ml consisted of containing 50mM phosphate buffer (pH 7.0), 0.5mM ascorbate and 0.5mM H_2O_2, the reaction was started with addition (H_2O_2). The (APX) activity was measured at 290nm. The enzyme activities of (CAT), (POD) and (APX) were expressed as µmol min^{-1} mg^{-1} protein.

Soluble Protein Content

The protocol of Bradford, (1976) was used to quantify soluble protein content in the enzyme extracts using Bovine Serum Albumin V as a standard.

Determination of Ascorbate Concentrations (AS)

Homogenized 0.5gm of fresh leaves in 5% (w/v) sulfosalicylic acid and then centrifuged at 10000 rpm for 10min. The reaction mixture was consisted of 2ml of 2% w/v sodium-molybdate, 2ml of 0.15mM NH_2SO_4, 1ml of 1.5mM $NaHPO_4$ and 1ml of extract. It was incubated at 60° C in water bath for 40min, cold and centrifuged again at 3000 rpm for 10min. The absorbance was measured at 660nm according to Kampfenkel *et al*., (1994).

Determination of Glutathione (GSH)

Grounded and dissolved 0.5gm fresh leaves in a mixture of 1ml of 25%. H_3PO_4 and 3ml of 0.1M sodium phosphate-EDTA buffer (pH 8.0). The homogenate was then centrifuged at 10000 rpm for 20min, and the supernatant was further diluted five times with sodium phosphate-EDTA buffer (pH 8.0). The final assay

mixture 2.0ml contained 100µl of the diluted supernatant, 1.8ml of phosphate-EDTA buffer and 100µl of O-phthalaldehyde 1mg ml^{-1}. After thorough mixing and incubation at room temperature for 15min, the solution finally transferred to a quartz cuvette and the fluorescence at 420nm was measured after excitation at 350nm according to Hissin and Hilf, (1976).

Determination of Non-Protein Thiol (NPT)

Homogenized 0.5gm fresh leaves in 5ml of potassium phosphate buffer (pH 8.0) in an ice bath, and then centrifuged at 10000 rpm for 20min. The supernatant was used for (NPT) assay using 5,5-dithio-2,2-dinitrobezoic acid as a reagent and the absorbance was at 412nm according to Metwally *et al*., (2003).

Statistical Analysis

Standard procedure of one-way ANOVA was used to analyze data, and the means were separated by Turkey's test. The differences indicated at $p \leq 0.05$ significance value.

RESULTS AND DISCUSSION

Khat leaves residues significantly decreased the content of chlorophyll **a** and **b** at the two tested concentrations (Figure 1). The loss in Chlorophyll **a** was 55.05% and 72.65%, while chlorophyll **b** was reduced by 38.80% and 65.00% at 25, 50gm of khat leaves residues, respectively. The applied (SA) was generally effective in partially or completely antagonizing the inhibitory effects of khat leaves stress on chlorophyll **a** and **b** content (Figure 1).

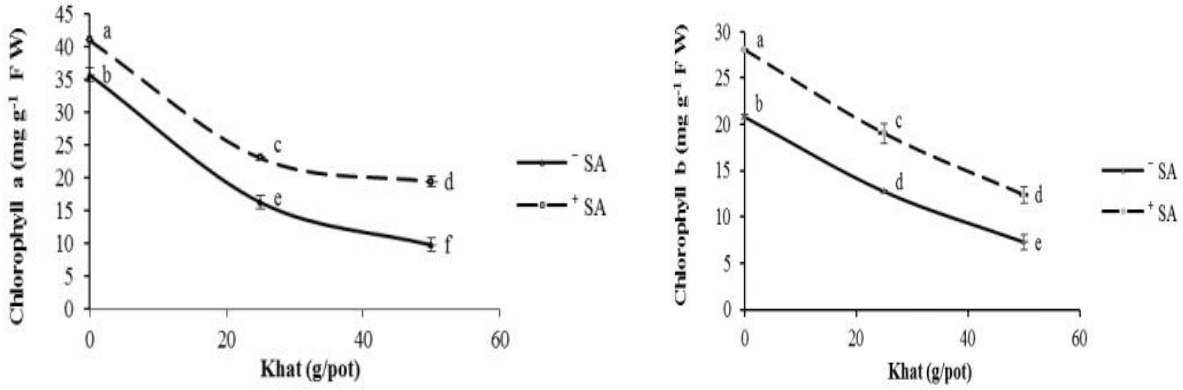

Figure 1: The Action of Salicylic Acid (SA) Treatment in Ameliorating the Adverse Effects of Khat Leaves Residues in Wheat Leaves on Chlorophyll a and Chlorophyll b of Wheat Leaves

* values in parentheses represent ±SD (n=5), ($P \leq 0.05$).

** Means which are not significantly different are followed by the same letter

It is well known that chlorophylls a and b represent the center parts of photosynthesis. The pronounced reduction of both chlorophylls a and b levels in wheat leaves after treatment with khat leaves residues compared with controls might be attributable to the presence of allelochemicals in khat leaves residues. Although, the exact mechanism for the reduction of chlorophyll content in plants treated by khat leaves residues is still not clearly known, but it is likely due to the inhibition of chlorophyll biosynthesis, the increase in chlorophyllase enzyme activity or both of them. The reduction of leaf chlorophyll content can also be correlated with an increase in the metabolic processes involved in the synthesis of new pigments (Natarajan and Elavazhagan, 2014; Nitesh and Ambika, 2016). The beneficial effects of the (SA) applied in partially or completely mitigating the adverse effects of khat leaves stress on chlorophylls were clearly exhibited by the test plants. This mitigation could be directly attributed to the role of applied (SA) in enhancing chlorophyll biosynthesis.

Khat leaves residues significantly increased (EL%) compared to control (Figure 2A). The increase in (H_2O_2) was 98.51% and 187.77% at 25, 50gm of khat leaves residues. (SA) pretreatment showed antagonizing partially or completely for the stimulatory effects of khat leaves residues on electrolyte

leakage (Figure 2A). This decrease was approximately estimated to be 36.15% and 22.80% at 25, 50gm of khat leaves residues, respectively. Also, pretreatment of (SA) alone decreased (H_2O_2) to 37.69%.

At 25, 50gm of khat leaves residues treatment, excised leaves of wheat plants showed an important accumulation of (H_2O_2) content (Figure 2B). The increase in (H_2O_2) levels was 1.51 and 2.01 fold for 25, 50gm of khat leaves residues, respectively. Pretreatment with (SA) significantly decreased (H_2O_2) levels in wheat leaves by 0.65 and 0.70 fold for 25, 50gm of khat leaves residues, respectively (Figure 2B). Under non-stress conditions added (SA) caused decreased (H_2O_2) levels in wheat leaves by 0.64 fold.

Lipid peroxidation measured as (MDA) content is therefore, an indicator of oxidative damage from stress. Compared to control, the level of (MDA) was significantly increased with increasing khat leaves residues (Figure 2C). The increase in (MDA) was 1.93 and 2.64 fold at 25, 50gm of khat leaves, respectively. Treatment of (SA) significantly decreased (MDA) level compared with treated plants (Figure 2C). The maximum decrease in (MDA) by the treatment of (SA) was 0.47 fold at 25gm of khat leaves and 0.33 at 50gm of khat leaves. Alone treatment with (SA) as well significantly decreased (MDA) level to 0.63 fold.

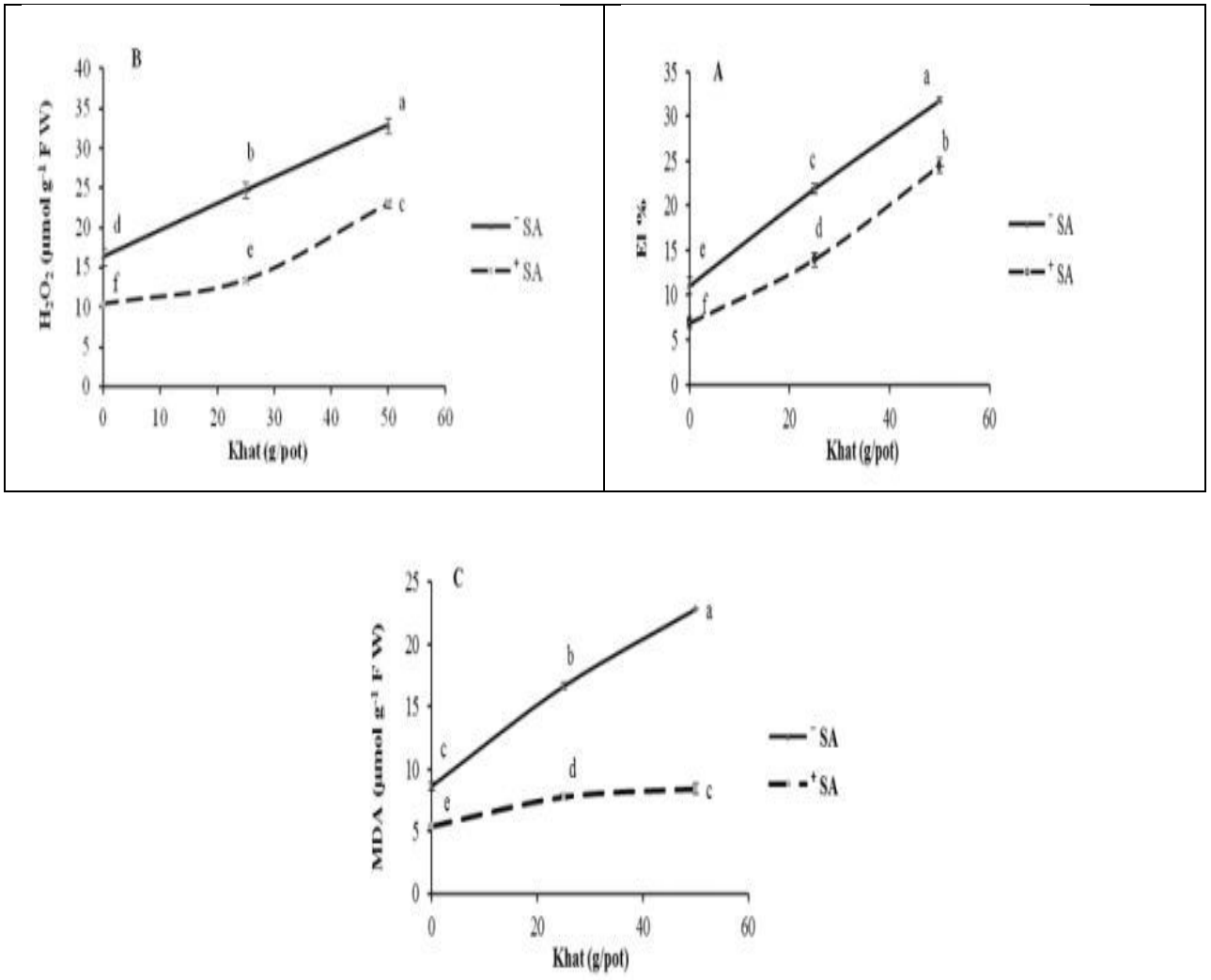

Figure 2: The Action of Salicylic Acid (SA) Treatment in Ameliorating the Adverse Effects of Khat Leaves Residues in Wheat Leaves; A: On Electrolyte Leakage (EL%); B: On Hydrogen Peroxidase (H_2O_2); C: On Malondialdehyde (MDA)

* values in parentheses represent ±SD (n=5), ($P \leq 0.05$).

** Means which are not significantly different are followed by the same letter.

Analysis of antioxidant enzymes (CAT), (AXP) and (POD) showed considerable changes in their activity by khat leaves residues (Figure 3A, B and C). Activity of (CAT) in wheat leaves was decreased to 17.21% and 42.32% at 25, 50gm of khat leaves residues, respectively compared to the control (Figure 3A). Pretreatment of (SA) significantly increased (CAT) activity to 105.97% at 25 gm and 145.46% at 50gm of khat leaves residues (Figure 3A). However, Pretreatment of (SA) alone also increased (CAT) activity to 25.47%. Similar trend was observed in the activity of (APX). The decreases were 35.98% and 45.33% with 25, 50gm of khat leaves, respectively. Pretreatment of (SA) initially increased (APX) activity in wheat leaves to 67.80 % and 58.42 % at 25, 50gm of khat leaves, respectively (Figure 3B). Also, (SA) alone increased (APX) activity to 40.36%. Likewise, (POD) activity was significantly increased by khat leaves residues (Figure 3C). Its increase was greater with 25gm (40.84%) than with 50gm of khat leaves residues (25.92%). Pretreatment of (SA) initially decreased (POD) activity in wheat leaves to 18.63% and 37.08% at 25, 50gm of khat leaves, respectively (Figure 3C). Also, (SA) alone decreased (POD) activity to 12.30%.

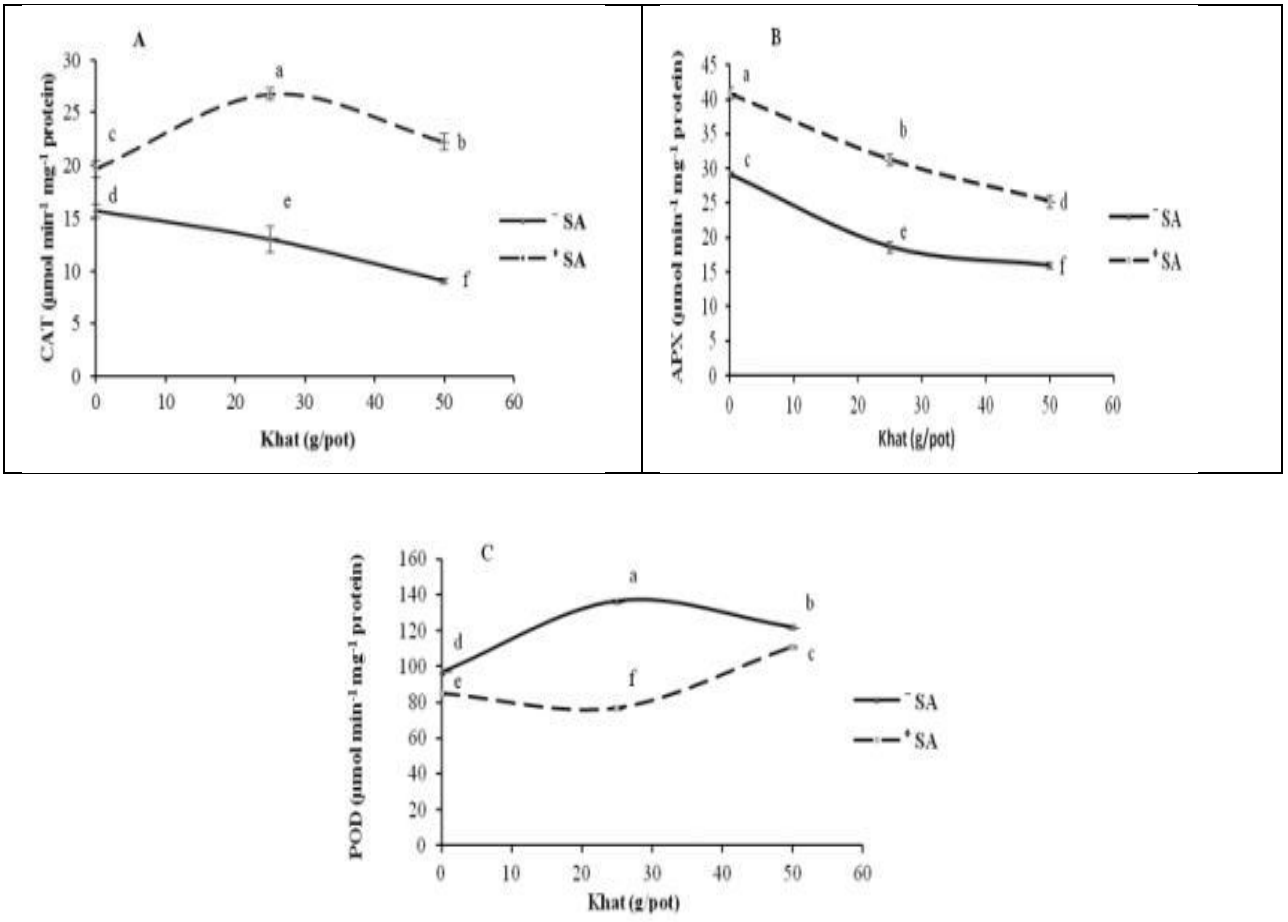

Figure 3: The Action of Salicylic Acid (SA) Treatment in Ameliorating the Adverse Effects of Khat Leaves Residues in Wheat Leaves;
A: On Catalase (CAT); B: On Ascorbic Peroxidase (APX); C: On Peroxidase (POD)
* values in parentheses represent ±SD (n=5), ($P \leq 0.05$).
** Means which are not significantly different are followed by the same letter.

Our electrolyte leakage results indicate that plasma membrane functions of wheat leaf cells were disrupted by khat leaves residues. Since, electrolyte leakage assay is a sensitive test for the identification of allelochemicals that destabilize cellular (H_2O_2) membranes (Chai *et al*., 2013) enhanced electrolyte leakage can also be an indication of the cell death induction (Mur *et al*., 2012). The present results indicate that (SA)

decreased oxidative stress, as clearly evident in the decreased electrolyte leakage of membranes correlating with the decrease of both (H_2O_2) and lipid peroxidation.

Likewise, it was observed that increasing khat leaves residues significantly enhanced the generation of (H_2O_2) in wheat leaves. (H_2O_2) is metabolized to H_2O by catalase and peroxidase. Such accumulation of the (H_2O_2) might rather be a result of lowered activities of (CAT) and (APX), responsible for scavenging (H_2O_2). Interestingly, the applied (SA) eliminated the accumulation of level by khat leaves residues. The lowering in (H_2O_2) levels under (SA) pretreatment is without doubt attributed to the increased activities of (CAT) and (APX) as a response to (SA) exposure. This result is in a good agreement with our previous study (Al-Mureish *et al.*, 2014).

Like (H_2O_2), (MDA) increased significantly in wheat leaves under khat leaves stress. The dramatic increase in (MAD) content was matched so well with the reduction in (CAT) and (APX) activities and the decrease in chlorophyll content under khat leaves stress. Generally, applied (SA) has partially or completely antagonizing for the stimulatory effect of khat leaves stress on (MDA) level of wheat leaves as reported earlier by Al-Mureish *et al.,* (2014). Generally, (SA) prevented (MAD) by oxygen free radicals, whether under khat leaves stressed or non-stressed control plants.

It is well known that (CAT) plays an important role in reducing oxidative stress by catalyzing the oxidant of (H_2O_2). In the present study, the results showed that the activity of (CAT) in wheat leaves was decreased under khat leaves stress. The decline in (CAT) activity could be attributed to (CTA) photoinactivation (Jiang and Huang, 2001) and inhibition of synthesis of new enzyme in the dark (Dat *et al.,* 1998), which may favor the accumulation of (H_2O_2) and consequently cause damage to cell membranes (Jankju *et al*., 2013).

(APX) is a key enzyme in the so-called ascorbate/glutathione cycle, which is a major hydrogen peroxide-detoxifying system in plant chloroplast and cytosol. APX-mediated detoxification of (H_2O_2) is coupled with ascorbate oxidation. Oxidized ascorbate is then regenerated via the oxidation of glutathione (Asada, 1992). The activity of (APX) was remarkably decreased with increasing khat leaves concentration as compared with the control. This reduction in (APX) activity could be associated with the decrease in (CAT) activity, which results in H_2O_2 accumulation (Al-Mureish *et al*., 2014). Mean while, remarkable increments were also observed in (CAT) and (APX) activities due to (SA) treatment. The increased activities of these enzymes helped the plants to destroy (H_2O_2) accumulated by khat leaves stress, which in turn led to elevate the plant tolerance to khat leaves stress.

(POD) activity was significantly increased by khat leaves stress. Since (CAT) activity was found to simultaneously decrease, this implies that (POD) should play a more significant role than (CAT) in detoxifying the produced (H_2O_2). Besides, it is well known that (CAT) is more less efficient than (POD) in scavenging (H_2O_2), because of its low substrate affinity (Erdal and Dumlupinar, 2011). The increase of (POD) activity under khat leaves stress might be an adaptive response and contribute to stress tolerance. However, the SA-mediated decrease in (POD) activity suggests its decreased (H_2O_2) scavenging ability. The decrease in (POD) activity might be associated with the increase of (CTA) and (APX) activities by (SA) pretreatment, which led to a lower (H_2O_2) content, and therefore there was less demand to activate the H_2O_2-scavenging enzymes. This may also suggest that (CAT) and (APX) play a more important role than (POD) in scavenging excessive (H_2O_2) in khat leaves stress.

Ascorbate (AS) content was markedly decreased by khat leaves stress (Figure 4A). The decrease in ascorbate induced by khat leaves treatment was found to be 38.44% and 57.25% at 25, 50gm of khat leaves, respectively. The adverse effects of khat leaves treatment on ascorbate content in leaves wheat was partially or completely by soaking seeds in (SA) (Figure 4A). The ameliorative effects of (SA) on khat leaves-induced reduction in ascorbate content were 72.97% and 96.37% at 25, 50g of khat leaves, respectively. (SA) alone showed remarkable increase in the content of ascorbate to 16.19%.

The addition of khat leaves consistently decreased (GSH) content in wheat leaves in both treatments (Figure 4B). The maximum decrease in (GSH) content was 32.14% and 54.46% at 25, 50gm of khat leaves, respectively. The applied (SA) was generally effective in partially or completely antagonizing the inhibitory effects of khat leaves stress on (GSH) content (Figure 4B). The maximum increase in (GSH)

content by (SA) treatment was 64.34% and 106.19% at 25, 50gm of khat leaves, respectively. (SA) alone significantly increased (GSH) content to 21.29%.
The content of (NPT) showed approximately 22.75% and 50.74% decreased in 25, 50gm of khat leaves stress (Figure 4C). The (SA) pretreatment initially stimulated (NPT) content in leaves wheat by 94.49% and 71.83% at 25, 50gm of khat leaves stress, respectively (Figure 4C). (SA) alone increased (NPT) to 27.23%.

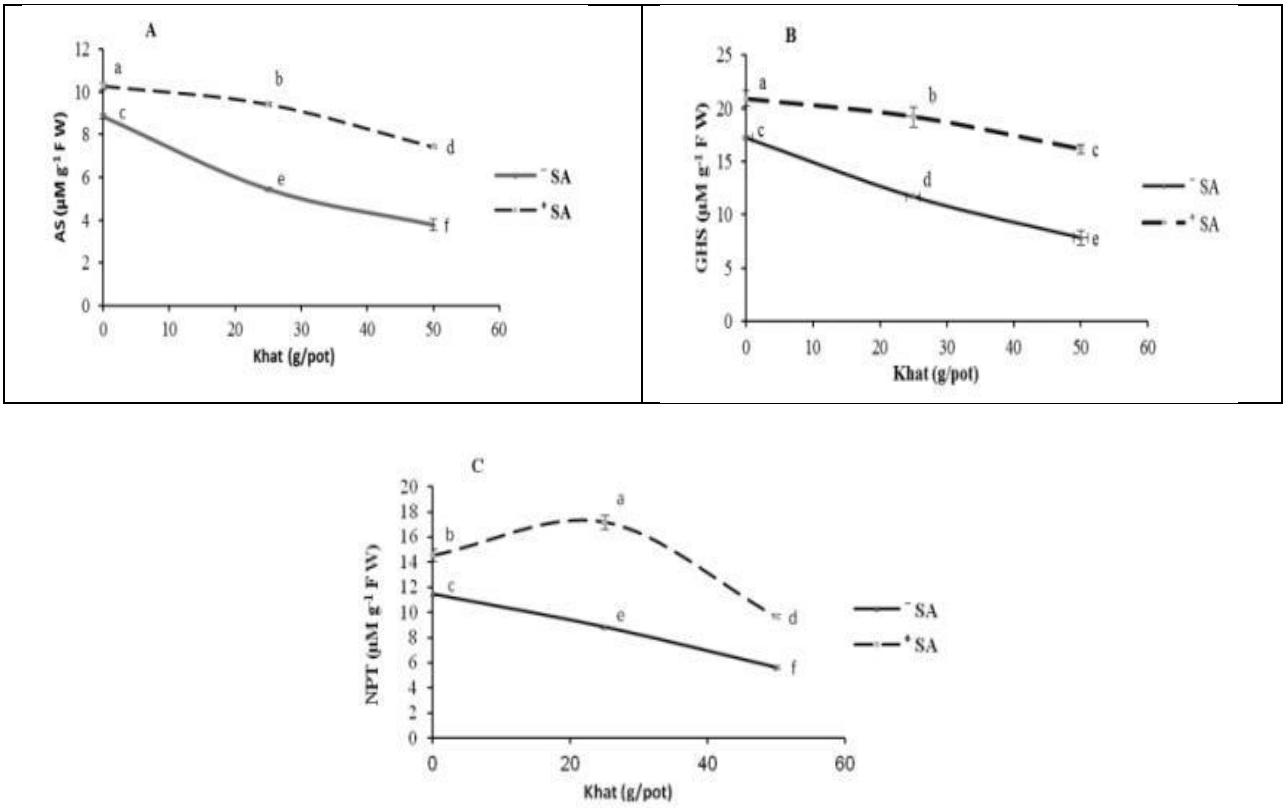

Figure 4: The Action of Salicylic Acid (SA) Treatment in Ameliorating the Adverse Effects of Khat Leaves Residues in the Wheat Leaves; A: On Ascorbate (AS); B: On Glutathione (GHS); C: On Non-Protein Thiol (NPT)
* values in parentheses represent ±SD (n=5), ($P \leq 0.05$).
** Means which are not significantly different are followed by the same letter.

Ascorbate (AS) functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress. Ascorbate can directly scavenge oxygen free radicals with and without enzyme catalysts and indirectly scavenge them by recycling tocopherol to the reduced form (Ebrahimian and Bybordi, 2012). Here, (AS) content was significantly declined in wheat leaves exposed to khat leaves treatment. Generally, the adverse effects of khat leaves stress on (AS) content in wheat leaves was partially or completely alleviated by soaking seeds in (SA). This result is also in a good agreement with our previous study (Al-Mureish *et al.*, 2014).
(GSH) is the major reservoir of non-protein thiols and plays an important role in the defense against ROS (Gill and Tuteja, 2010). It can be noted that the addition of khat leaves residues consistently decreased (GSH) content compared with the control. Reduced (GSH), a disulphide reductant that protects thiols of enzymes and reacts with singlet oxygen (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-) together with ascorbate, plays a pivotale role in protecting the plants from the ROS (Noctor and Foyer, 1998). The decline in the levels of (GSH) might be attributed to a decreased glutathione reductase activity (Dixit *et al.*, 2001). Pretreatment with (SA) significantly alleviated the (GSH) caused by khat leaves stress.

The level of (NPT) in wheat leaves was significantly decreased by khat leaves residues. Pretreatment with (SA) significantly enhanced (NPT) level in leaves of wheat with or without khat leaves treatment. On the bases of this criterion, we demonstrated that wheat leaves pretreated with (SA) showed a significant protection against the subsequent khat leaves stress. This protective effect has also been described earlier (Al-Mureish *et al*., 2014).

The pretreatment of (SA) alleviated khat leaves-induced oxidative stress as evidenced by the decrease in concentrations of (MDA) and (H_2O_2), electrolyte leakage (EL%) and (POD) activity. Pretreatment of (SA) stimulated the inhibitory role of khat leaves stress on (AS), (GSH), (NTP), (AS) contents, activities of (CAT) and (APX) in the treated plants with and without khat leaves residues. It is interesting to conclude that the adverse effects of khat leaves residues in enzymatic antioxidants involved in the oxidative defense mechanism in plants can be significantly alleviated by application of (SA).

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