Antioxidative Effects of Acetone Fraction and Vanillic Acid from *Chenopodium murale* L. on Tomato Plant

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Abstract: From the active acetone fraction of *Chenopodium murale* L., vanillic acid has been isolated and identified based on ^{1}H and ^{13}C NMR spectral analyses. Free phenolic compounds inside the active acetone were qualified and quantified by using HPLC analysis which revealed the presence of seven compounds with abundance of vanillic and *p*-hydroxybenzoic acids. The allelopathic potential of the acetone fraction and vanillic acid has been evaluated through laboratory bioassays against tomato plants. Our results showed that allelopathic potential induced by low concentrations of acetone fraction and vanillic acid stimulated germination and growth of tomato and had stimulating effects on the activity of some anti-oxidant enzymes. We observed an enhancement in the activities of catalase, peroxidase, superoxide dismutase and polyphenol oxidase. Meanwhile, the levels of free phenolic compounds, H_2O_2 and lipid peroxidation were decreased. The highest stimulations were recorded at 50 ppm (acetone fraction) and 0.5 ppm of (vanillic acid). In contrast, the highest concentrations exerted negative effects on all the measured parameters to record the maximum value of inhibition at 400 ppm (acetone fraction) and 4 ppm (vanillic acid). Furthermore, the results stated that bio-activity of active acetone was greater than active acetone. Collectively, at low concentrations our results proved the antioxidive effects of active acetone and vanillic acid and its potent use as stimulator for tomato germination and growth.
Keywords: *Chenopodium murale* L., Vanillic acid, Acetone fraction, Chromatography, Chromatography, Antioxidative effects

1. Introduction

Allelopathy is a natural and an environment-friendly technique that may prove to be a unique tool for enhancement of crop yields, and improving the ecological environment [27,29]. Many studies found that some weed extracts may be used as bioherbicides to control the germination and growth of other weeds [14,19]. Moreover, some weed residue possesses allelopathic potential to increase the growth and yield of some crops [48]. *Chenopodium album* (family: chenopodiacea) one of the weeds that possesses allelopathic potential to stimulate growth of some crops [28].

Plant phenolic compounds are among the major allelochemicals implicated in allelopathy [18,6,9,8]. The production of secondary metabolites, especially phenolic compounds, are essential for plant survival and can play a direct role in self-defense and the plant protection [41,22]. Recent studies stated that low concentrations of phenolic compounds such as, Feurlic, *p*-hydroxybenzoic coumaric and galic acids induced the plant to produce phenolic glycosides [15], and it may be effective in plant protection [24]. Phenolic acids such as, caffeic, ferulic and vanillic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables, and other plants. [40]. Besides, Hegab [15] and Baziramakenga *et al.* [5] reported the stimulation of protein synthesis and activation of antioxidant enzymes with the application of phenolic acids at low doses. The high ability of phenolic constituents to neutralize the active oxygen species is strongly associated with their structure, such as the conjugated double bonds and the number of hydroxyl groups in the aromatic ring, mostly attributed to flavonoids and cinnamic acid derivatives [31,44]. Additionally, anti-oxidative effects of phenolic acids depend upon their contents through different sample preparations [26], extraction [55] and hydrolysis procedures [33]. Oppositely, phenolic acids at high levels suppressed activities of many antioxidant enzymes and increased lipid peroxidation [35,15]). Vanillic acid is one of the phenolic acids that was identified in many plants such as *C. murale* [3], *Melilotus messanensis* [28], *Juglans regia* L. [54], and *orchard grass* [34]. Vanallic acid may be used as a natural antioxidant instead of synthetic ones which have a toxic potential [54].

Chenopodium murale (nettle-leaved goosefoot; family Chenopodiaceae) is an annual problematic weed found extensively in the arable lands [16]. A native of Europe, the weed has established in various parts of the world including Egypt [25,42] and India [16]. Field observations reveal that C. murale competes with crops and causes reduction in crop qualities and yields. [1,16]. Batish *et al.* [3] stated that root exudates and residues of *C. murale* significantly affect the wheat growth by contributing phenolic allelochemicals in the soil rhizosphere. Then they found 4 phenolic acids (vanillic, benzoic, ferulic and *p*-coumaric acids) in this weed root residue amended soils. Although *C. murale* causes serious interferences in agricultural production, little information on allelochemicals from shoot extract of this weed has been documented, Therefore, we conducted this research to (a) determine the possible allelopathic potential of different organic solvents extracts from shoots of *C. murale* and choose the most active one (b) identify the active compounds of the selected extract and isolate the most active one (c) bio-assay the allelopathic effect of the most active extract and

the isolated compound on germination, growth, and some metabolite contents of tomato seedlings, as well as, their effects on some anti-oxidant enzymes activities.

Materials and Methods

Plant Material

Mature nettle-leave goosefoot, (*Chenopodium murale* L.), was collected from different cultivated fields around Beni-Seuf city during fruit development stage in 2008. Shoot of the target weed were dried for 4 weeks at room temperature. The dried shoot were ground and stoked in plastic sacs in dark condition at room temperature until use.

Extraction and Separation

Air-dried leaves (100 g) of *C. murale* were extracted with 75% ethanol solution with shaking of suspension for 24 hours on a shaker (200 rpm) at room temperature. The extract was centrifuged at 4000 rpm then filtrated through Buchner funnel. The filtrate was concentrated with a rotary evaporator under reduced pressure at 45° C under reduced pressure to remove ethanol. The resulting suspension was successively partitioned with hexane (2.43 g), acetone (7.56 g) and n-butanol (3.71 g).The hexane (F1), acetone (F2) and *n*-butanol (F3) fractions were separately combined and evaporated to dryness under reduced pressure, while the aqueous layer (F4) was lyophilized to dryness to yield 5.19 g and the yield of all fractions was 18.89 g. All the fractions were tested using wheat coleoptiles bioassay.

Coleoptiles Wheat Bioassay

Wheat grains (*Triticum aestivum* L. cv. Sides) were sown in Petri dishes and incubated darkly at 22±1°C for 4 days [13]. The apical 2 mm of the shoots were cut off and discarded, and then the next 4 mm of the coleoptiles were taken under a green safelight for bioassay. Four different concentrations for every fraction and sub-fractions were diluted in a phosphate–citrate buffer solution containing 2% sucrose at pH 5.6 to prepare 10, 50, 100 ppm (the experimental solutions) [32]. Five wheat coleoptiles were placed in test tube containing 4ml of the experimental solution (three replicates of each experiment) and control tubes were filled with 4ml of the buffer solution. All test tubes rotated slowly for 24 h at 22±1°C in dark condition. The coleoptiles length was measured and the data expressed as percentage of differences as compared with the control in order to see whether the extracts had an additive or negative effect on the growth.

Isolation of the Most Active Compound

The acetone fraction (F2) was found to be the most active. Therefore, this fraction was subjected to repeated column chromatography on a silica gel column and the mobile phase (hexane: ethyl acetate) was chosen for elution depending up on TLC analysis. The active fraction was eluted stepwise with hexane with an increasing amount of acetone to give 11 sub-fractions (F2-1 to F2-11). All those Sub-fractions having different activities and the most biological activity was found in F2-8. The active fraction, F2-8 (105 mg) was further purified on a silica gel column using chloroform- acetone as the eluting solvent, which resulted in the isolation of vanillic acid (83 mg) as the major active compound.

NMR Identification

The isolated pure compound from F2-8 was identified based on spectroscopic analysis, and on comparison of ^{1}H and ^{13}C NMR data with literature values.

Preparation of Test Solutions

Both active acetone extract fraction and identified allelochemicals from *C. Murale* leaves were tested at different concentrations. The active acetone fraction was dissolved in very small amount of DMSO, and then diluted with distilled water to prepare the experimental solutions (10, 50, 100, 200 and 400 ppm), The isolated pure compound (vanillic acid) was used to prepare the test solution (4 ppm) by dissolving it in a little amount of DMSO and then was completed with distilled water to the exact concentration and the rest (0.1, 0.5, 1, 2, and 4 ppm) were obtained by dilution of 4 ppm. In the same way controls were performed.

Tomato Germination and Growth Bioassay Experiments

The further studies on activity of suspected biologically active materials were evaluated in seeds germination and seedlings growth tests of tomato. Tomato seeds were surface sterilized in a solution of 10% (v/v) H_2O_2 for 10 min, washed thoroughly for three times in distilled water then were soaked in test solutions for 12 hours. After soaking, treated seeds (100) of each test solutions were sown in sterilized 9-cm glass Petri dishes, then irrigated with 10 ml of aqueous solutions at controlled pH by using 10−2 M 2-[*N*-morpholino]ethanesulphonic acid (MES) and addition of solution of NaOH 1 M (pH 6.0). Each treatment was replicated three times. The Petri dishes were incubated in dark germinating chamber for 10 days at 25/12ºC ± 2 day/night and 97% relative humidity. The same bioassay was performed for untreated seeds to serve as control.

The Activity of Anti-oxidant Enzyme bioassay

Fresh seedlings of tomato were extracted with 2.5 ml of 67 mM cold phosphate buffer (pH 7.0) as described by Shann and Blum [43]. The homogenates was centrifuged at 10000 rpm for 15 min at 4 ºC.

Catalase (CAT) (EC 1.11.1.6) was assayed by measuring the initial rate of disappearance of H_2O_2 [21]. The decrease in H_2O_2 was followed as a decline in the absorbance at 240 nm and the activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm). The activity was expressed in units of µM of destroyed H_2O_2 min^{-1} g^{-1} fresh weight. Peroxidase (EC 1.11.1.7) activity was measured according to Kar and Mishra [20]. The enzyme activity was expressed as the change in the optical density of pyrogallol min^{-1} g^{-1} fresh weight. Polyphenoloxidase (POL) (EC 1.10.3.1) activity was measured according to [20]. Enzyme activity was expressed as the change in the optical density of pyrogallol min^{-1} g^{-1} fresh weight. Superoxide dismutase (SOD)(EC 1.15.1.1) activity was determined according to the method of Beyer and Fridovich, [7]. One unit of SOD activity was defined as the amount of enzyme required to cause inhibition of the photo-reduction of NBT by 50%. (U mg^{-1} FW).

Determination of H_2O_2 content and Lipid Peroxidation (LP)

H_2O_2 level was determined as per the method of Velikova *et al.* [50]. The amount of H_2O_2 was computed from the standard curve made earlier with known concentrations of H_2O_2 and expressed as nmol g^{-1}FW. Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for determining the extent of lipid peroxidation. According to Preuss *et al.* [37] lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reactive substances (TBARS) and expressed as n mol malondialdehyde (MDA) (nmol g^{-1} FW) using the extinction coefficient (156 mM^{-1} cm^{-1}).

Determination of the Proline Content

Proline was determined following Bates *et al.* [2] PRO content was determined from a standard curve. It was expressed as mg g^{-1}DW.

HPLC Analysis for Free Phenolic Compounds in AF

A mixture of twenty standard phenol compounds of high purity was used for HPLC analysis. HPLC analysis was based on the comparison of the retention time of a mixture of standard phenolics with those in plant samples. The Dried acetone extract of *C. murale* was dissolved in HPLC grade MeOH to give 1000 ppm. Methanol dissolved sample was filtrated through 0.2 µm Millipore filter before HPLC analysis, then 20 µl of it was injected into HPLC.

Statistical Analysis

The experimental design was completely randomized with three replications. The results of bioassay experiments were analyzed with one-way analysis of variance and the mean values were separated at $P < 0.01$ and $P < 0.05$. The statistical analysis was done using the SPSS® / PC computer software package version 11.1., 2001.

Results and Discussion

Different Solvents Fractionations and Isolation of the Most Active Compound

The fine powder of dried shoots of *C. murale* was extracted with 80% aqueous ethanol to yield about (18.89 g), then it was sequentially fractionated with solvents of different polarities to obtain hexane, acetone, n-butanol and aqueous fractions. Different organic solvents' fractions were bio-assayed using wheat coleoptiles. Among the four fractions, acetone fraction (AF, 7.65 g) showed the greatest stimulatory bioactivitiy compared with the control. AF was fractionated using silica gel column and TLC chromatography. Further, bioassay experiments were carried out on the obtained 11 sub-fractions to determine the most active one. The results reported F2-8 (105 mg) as a most active sub-fraction. Repeated silica gel column chromatography was conducted to purify the active sub-fraction and isolate the most active compound (VA, 83 mg).

Structure Elucidation for Isolated Compound

The identification of isolated active compound was performed with ^{1}H and ^{13}C NMR and the data were given as follows: ^{1}H NMR (MeOH-D_4): δ 3.91 (3H, s, OCH_3), 7.12 (1H, d, J = 8.2 Hz, H-5), 7.57 (1H, d, J = 2.0 Hz, H-2), 7.63 to 7.65 (1H, dd, J =8.3 and 2.0 Hz, H-6. And ^{13}C NMR (MeOH-D_4): δ, 55.4 (O-CH_3), 112.2 (C-5), 115.3 (C-2), 123.8 (C-1), 125.2 (C-6), 147.6 (C-3), 151.6 (C-4), 169.0 (COOH)]. Comparing the above data with previous results that were reported by Huang *et al.* [17], Zhang *et al.*, [54] and Termentzia *et al.* [46] that helped us to identify our isolated compound as vanillic acid (VA).

HPLC Analysis

In HPLC analysis, each compound was identified by peak area measurement relative to the standard peak area at 254 nm (Table 1). Seven phenolic compounds (vanillic, *p*-hydroxybenzoic, cinnamic, caffeic, protocatechuic, ferulic and *p*-coumaric acids) were identified in the AF of *C. murale* shoots. Similarly, Batish *et al*. [3] reported that upon HPLC analysis of the *C. murale* root residue-amended soils, vanillic, ferulic, and *p*-coumaric acids were identified. Vanillic and *p*-hydroxybenzoic acids recorded the major amounts (48.6, and 24.4%, respectively) of the total concentration of determined free phenols, while cinnamic, caffeic and protocatechuic acids were found at lower amounts (0.6, 1.2, and 3.6, respectively). The other compounds, including ferulic and *p*-coumaric acids, accounted about 14.7 and 6.9%, executively.

Table 1. Identification of free phenolic compounds in acetone extract of *C. Murale* shoots using HPLC analysis

Standard phenolic compounds	Retention Time (min)		Concentration (μg g^{-1} dry weight)
	Standard	Sample	
Protocatechuic acid	13.919	14.106	78.12
p-Hydroxybenzoic acid	16.032	16.156	528.32
Vanillic acid	18.379	17.850	1053.5
Caffeic acid	19.61	19.196	25.63
p-Coumaric acid	23.208	22.832	148.68
Ferulic acid	23.951	24.021	318.776
Cinnamic acid	36.149	35.851	12.53
Total concentration			2165.556

Germination and Growth Bioassay

The effect of AF and VA was concentration dependant (Fig. 1, 2). In the context, phenolic compounds (as coumarin) were able to promote or inhibit plant growth, the response being species-specific and concentration-dependent [30]. Low concentrations of AF (10, 50, and 100 ppm) and VA (0.1, 0.5 and 1.0 ppm) stimulated the germination and growth of the

tested tomato, while higher concentrations had slight inhibitory effects. These results agreed with that of Reigosa *et al.* [39] who reported the inhibitory effects of several phenolic compounds (ferulic, gallic, *p*-coumaric, *p*-hydroxybenzoic, vanillic acids and *p*-vanillin) on the germination and seedling growth of different weeds, whereas lower concentrations were of stimulatory effects. Phenolic acids have been identified as allelopathic agents, which includes both positive and negative effects [22]. The concentration (50 ppm) of AF recorded the highest stimulatory values of germination, as well as, plumule and radicle growth (22.25, 39.41 and 25.42%, as compared to the control, respectively). At 0.5 ppm, VA had also similar significant stimulatory effects (18.65, 31.58, and 32.56%, respectively). In the same manner, the treatment with vanillin and mixture of phenolic compounds at lower concentrations produced enhancing effects on germination and growth of pea seeds [15].

As the concentration increases, the inhibitory effects increase. For instance, at 400 ppm of AF, germination of tomato was inhibited by (14.2 %) and at 4 ppm of VA, the inhibition value was (11.14 %). These results coincided with the findings obtained by Souto *et al.* [45] who revealed that germination of *Trifolium repens* seeds was inhibited at high concentrations of all allelochemicals utilized (*p*-vanillin, gallic, vanillic, ferulic, *p*-hydroxybenzoic and *p*-coumaric acids) applied. Conclusively, stimulatory and inhibitory effects of AF were much greater than those of VA. The higher effects of AF could be attributed to the possible synergistic effect between its allelochemicals content [39,15]. Moreover, roots seemed more sensitive to the allelopathic extracts than shoots, the result endorsed earlier approaches which reported that root growth is more sensitive to extracts than hypocotyl growth [10]. Variability in root and shoot sensitivity might have been caused by the fact that roots were in direct contact with the extract and subsequently with inhibitory chemicals as described in earlier works with various crops and weeds [38].

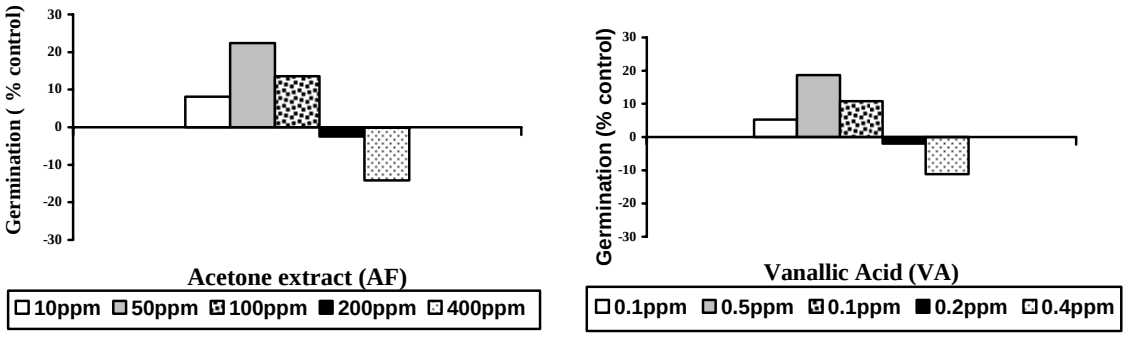

Figure 1. Effect of AF and VA of *C. murale* at different concentrations (ppm) on the germination of tomato as percentage over or below the control (Values are the mean of three replicates).

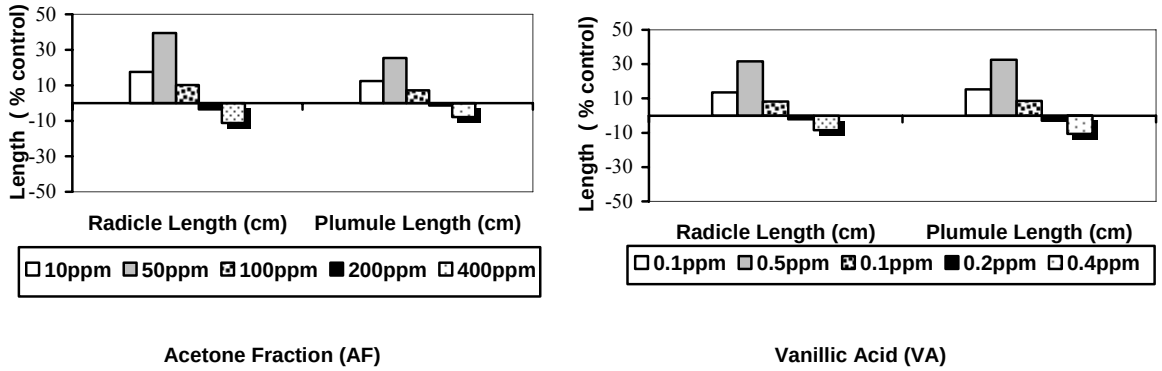

Figure 2. Effect of AF and VA of *C. murale* at different concentrations (ppm) on the radicale and plumule length of tomato seedlings as compared as percentage over or below the control (Values are the mean of three replicates).

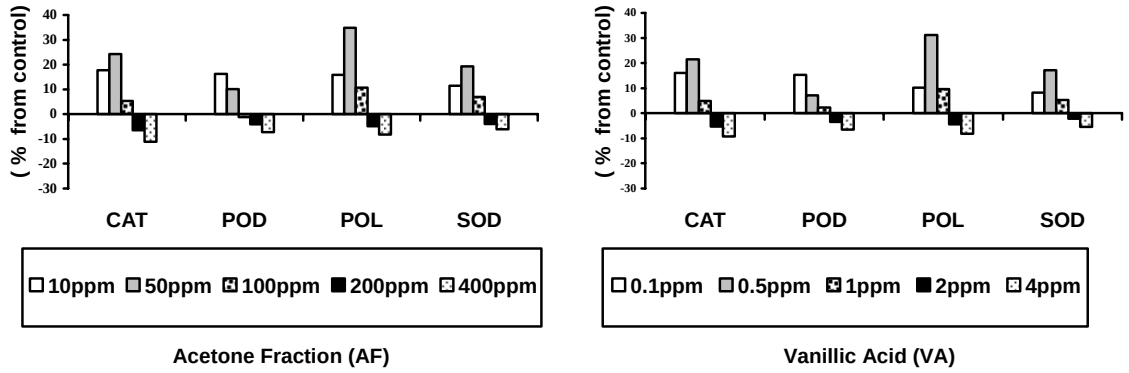

Figure 3. Effect of AF and VA of *C. murale* at different concentrations (ppm) on activities of catalase (CAT) (µM H_2O_2 destroyed min^{-1} g^{-1} FW), Peroxidase (POD) (A420, µM Purpurogallin min^{-1} g^{-1} FW), polyphenoloxidase (POL) (A420, µM Purpurogallin min^{-1} g^{-1} FW), superoxide dismutase (SOD) (U g^{-1} FW).

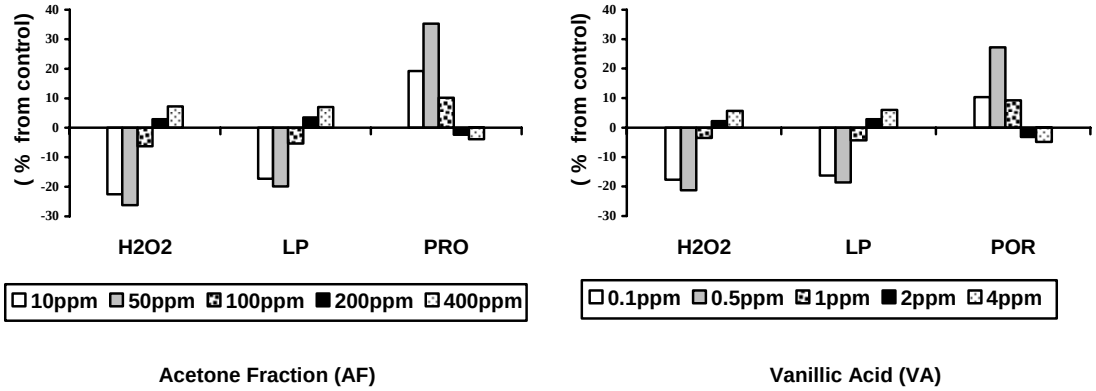

Figure 4. Effect of AF and VA of *C. murale* at different concentrations (ppm) on lipid peroxidation (LP) (nmol MDA g^{-1} FW), H_2O_2 (nmol g^{-1}FW) and POR (mg g^{-1}DW) content were determined as percentage over or below the control (Values are the mean of three replicates).

Antioxidant Activity

Dependent on their concentrations, AF and VA could induce both stimulatory and inhibitory effects on the antioxidant activity system of tomato. Measurement of the anti-oxidative enzymes activities in response to all treatments indicated a stimulatory pattern at low concentrations and an inhibitory pattern at high concentrations (Fig. 3). The activities of CAT, POD, POL and SOD were stimulated by AF and were markedly high at 50 ppm (24.3, 1.1, 34.9, and 19.25%, as compared with control, respectively). This increase could be attributed to the ability of plants to improve the scavenging system [49]. Similarly, the data clearly recorded enhancement in measured activities of CAT, POD, POL and SOD at 0.1, 0.5 and 1 ppm of VA with maximum stimulation (21.52, 7.15, 31.25 and 17.21%, respectively) by (0.5 ppm). The marked increases in anti-oxidant enzymes (CAT, POR, and SOD) have also been observed in other studies on modes of action of allelochemical such as, phenols [52,36]. On the other hand, the higher concentrations of AF and VA suppressed the activities of anti-oxidant enzymes. The highest concentration (4 ppm) of VA caused a maximum degree of inhibition of CAT, POD, POL and SOD activities (9.25, 6.55, 8.14, and 5.42 %, compared below control, respectively). Comparatively, AF had more inhibitory effect than VA at high concentrations.

Foyer *et al.* [11] envisaged H_2O_2 as central components of signal transduction in both environmental and abiotic stresses in plants, as well as, its level correlates to activities of CAT. Both of these enzymes consume hydrogen peroxide although they showed inversely correlated activities. Tomato seedlings, at application of low concentrations, showed low

levels of H_2O_2 which decreased to minimum value with AF at 50 ppm and VA at 0.5 ppm (24.24 and 21.2% below control, respectively) (Fig. 4). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Noctor & Foyer 1998). The radical-scavenging activity of polyphenols depends on the molecular structure and the substitution pattern of the hydroxyl groups, the availability of phenolic hydrogens, and the possibility of stabilisation of the resulting phenoxyl radicals via hydrogen donation or by expanded electron delocalisation [6]. Upon the increase in applied concentrations, the reduction in H_2O_2 content was followed by a slight stimulation up to 7.18 and 5.68%, respectively causing oxidative stresses. The toxicity of many quinones and phenols can largely be attributed to the formation of semiquinone radicals that donate electrons to molecular oxygen, forming superoxide anions ($O_2^{\bullet-}$) [47]. These can undergo a series of further reactions to become the more reactive hydroxyl ($OH^{\bullet}$) or hydroperoxyl ($HO_2^{\bullet}$) radicals [12]. Furthermore, results by Zeng *et al.* [53] indicated a significant reduction of SOD and POD activities which may cause a mass accumulation of active O_2 in plant leaves. We also measured the changes in lipid peroxidation which serves as an indicator of the extent of oxidative damage under stress by allelochemicals [51]. To determine if allelochemicals causes oxidative damage in tomato, we monitored changes in lipid peroxidation by measuring the amount of malondialdehyde (MDA) accumulation (Fig. 4). The low levels of concentration caused inhibition in accumulated MDA which reached maximum by AF (19.85%) at 50 ppm and VA (18.55% at 0.5 ppm). These results agreed with Hegab *et al.* [15] who stated that VA at low concentrations decreased the MDA content and prevent lipid proxidation. By increasing concentrations, the accumulation of MDA showed slight increase by 7.05 % of and 5.99 % for the applied AF (400 ppm) and VA (4 ppm) respectively. With respect to lipid peroxidation, Baziramakenga *et al.* [4] also reported that benzoic and cinnamic acids induced LP, which resulted from free radical formation in plasma membranes. Also the treatment of cucumber roots with *p*-coumaric acid at high level caused an increase of LP in association with the deterioration of membrane integrity [35].

Amount of endogenous free PRO increased significantly in response to different concentrations of AF and VA (Fig. 4). The lower concentrations of AF and VA increased PRO content than that in control. At 50 ppm of AF and 0.5 ppm of VA, the increases in PRO were 35.23 and 27.25% over their respective controls Khedr *et al*. [23] opined that PRO plays an important role as antioxidant molecule and/or would stimulate the activity of the antioxidative enzymes involved in defense mechanism against oxidative stress. Meanwhile, at higher concentrations, PRO content slightly decreased to reach their maximum value of inhibition at 400 ppm of AF and 4 ppm of VA.

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